A PHYLOGENETIC STUDY OF CARDIAC ANTIGENS

SUMMARY

THESIS

For

DOCTOR OF MEDICINE

(PATHOLOGY)

BUNDELKHAND UNIVERSITY,

JHANSI (U. P.)

1989

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SUMMARY AND CONCLUSIONS

In the present study, various antigenic systems contained in the heart of different species of animals viz. Fish, Frog, Tortoise, Hen, Bat and Pig have been investigated, and attempts have been made to analyse the phylogenic development of these antigens and also to find out any possible immunological correlation with reference to number of cardiac antigens and complexity of development of heart, in these animals as the heart became from two to four chambered right from pisces to mammalia, during the course of phylogenic development.

Rabbits were immunized heterologously with cardiac antigens (suspension of heart extract and complete Freund's adjuvant in equal proportion) of various species of animals viz, Fish, Frog, Tortoise, Hen, Bat and Pig, once a week for six weeks consequetively. Sera from rabbits were collected ten days after the last injection and tested for the presence of anticardiac antibodies using agar gel diffusion (AGDP), immuno-electrophoresis (IEP) and immuneprecipitation electrophoresis (IPE) techniques.

Sera of all the rabbits immunized with different cardiac antigens reacted with the development of variable number of precipitin bands against corresponding cardiac antigen in AGDP, IEP and IPE techniques. Thus anti-fish-heart

antiserum and anti-tortoise-heart antiserum reacted with the corresponding cardiac antigens to give only two precipitin bands by all these techniques whereas the anti-frog-heart antiserum, anti-hen-heart antiserum and anti-bat- heart antiserum gave three precipitin bands against the corresponding cardiac antigens; the pig heart extract gave four to five precipitin bands against corresponding cardiac antigen.

The cross-reactivity of anti heart antisera of various animal species with the cardiac antigen of different animals as well as in reverse experiments the anti-cardiac antisera reacted only with the cardiac antigen of the corresponding animal species (immunizing species), and not with the cardiac antigen of any other species of animals. It would suggest that common cardiac antigens are not shared by these animals and the antigenic system contained in heart of different species of animals are species specific.

The results of all the three techniques viz. AGDP, IEP and IPE were identical in all experiments, therefore in some of the further experiments only one or two of these techniques were used for the detection of cardiac antigens.

It is conceivable from the results obtained after exposure of various heart antigens to various temperatures that fish heart antigens are heart labile and are completely

destroyed at 70°C or more within 30 minutes. The frog heart antigen could withstand 70°C temperature upto 30 minutes whereas it became inactive at 80°C temperature or more. The tortoise heart antigen was extremely heat labile and destroyed even after exposure to 55°C temperature. Hen heart antigens were much resistant to heat as evident from the results that it could withstand temperature upto 100°C, reactivity of hen heart antigen, though faint, remained intact even after exposure to 100°C temperature. The heart antigens contained in bat were moderately heat labile and were found inactive at 70°C temperature or more. The pig heart antigens are the most resistant to higher temperature and the reactivity of some of the antigenic fractions remained intact even at 100°C temperature.

The heart extracts variously exposed to pH, on cross reactivity with corresponding anti heart antiserum in AGDP, IEP and IPE techniques revealed that fish heart antigens were optimally active at moderately alkaline pH 7.2, 8 and 9.5, whereas the antigenic reactivity was lost at a low pH of 4.5, Tortoise heart antigen was much sensitive to change in pH; it was labile at low (4.5 and 6) as well as high pH (8 and 9.5) and was found to be optimally active only at pH 7.2. The hen heart antigen was moderately active at acidic pH 4.5 and 6, optimaly active at 7.2 and

labile at pH 9.5. The bat heart antigenic systems were moderately active at pH 4.5, 6 and 9.5 whereas optimally active at pH 7.2. The pig heart antigens were feebly reactive at acidic pH 4.5 and 6 while moderately active at pH 8, and optimally active at 7.2, whereas it was labile at pH 9.5.

The results of cross reactivity of protein fractions obtained after graded salting out with ammonium sulphate and the corresponding anticardiac antiserum revealed that the fish heart antigens were partially isolated at 40% and 60% saturation with ammonium sulphate whereas at 100% saturation all components were isolated indicating thereby that fish heart antigens are protein in nature. The frog heart antigens were partially isolated at comparatively lower saturations with ammonium sulphate whereas all components were precipitated at 100% saturation, which indicates that these are also protein in nature. All the antigenic components contained in tortoise, hen and bat heart were also precipated at 100% saturation with ammonium sulphate which indicates that all the antigenic fractions contained in tortoise, hen and bat heart are also protein in nature. However, even at 100% saturation with ammonium sulphate only three out of 4 to 5 tigenic fraction of pig heart could be isolated which

indicated that beside three protein antigens, atleast one to two antigenic fractions contained in pig heart could be other than protein.

Previous studies suggested that heart polysaccharide is antigenic acting like hapten (Chaturvedi, Gupta and Mehrotra et al 1971, Gupta 1977). The results reported here are not inconsistent with the possibility that one to two antigenic fractions of pig heart are polysaccharide in nature.

The relevant literature on the subject has been r iewed and the findings of the present work discussed in light of it.

C E R T I F I C A T E

It is to certify that the work entitled "A PHYLOGENETIC STUDY OF CARDIAC ANTIGENS" being submitted as thesis for M.D. (Pathology) examination of Bundelkhand University, 1989 by DR. DVIJENDRA NATH has been carried out in the Department of Pathology, under our guidance and supervision. The techniques and methods described in this thesis have been under taken by the candidate himself and the observations recorded have been periodically checked by us.

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ACKNOWLEDGEMENT

The completion of my work has provided me an opportunity to express my heartfelt gratitude to those who have been the part and parcel of this work and brought it to the completion. The feelings of gratefulness dwelling inside my heart can not be expressed in words and of course I realize that "The tongue is silent when heart is full".

It is my profound pleasure to express eternal indebtedness to a renowned and vivific personality, paragon of academic character and benevolence Dr. R.K.Gupta, M.D., M.N.A.M.S., Professor & Head of the Department of Pathology M.L.B. Medical College, Jhansi, whose inspirations and constant stimulus fruitfully resulted in the completion of this humble venture. As a senior thoughtful Pathologist, the words which he penned, reflect his charisma and importance he attached in carryingout this hardsome task for me. His valuable guidance moulded this work into a fine illustrated shape to see the light of the day.

The words are inadequate to express my pious reverence and the deepest sense of gratitude to the learned and vivacious physiologist Dr.(Mrs.) Sushma Gupta, M.D., Senior Lecturer in Physiology, M.L.B. Medical College,

Jhansi, who provided me an opportunity to work under her supervision. The present work at every stage bears the impression of her wise and concrete guidance, constructive criticism and meticulous attention.

I have privilege to pay my sincere regards to Dr. V.K.Sharma, M.D.,D.C.P., Lecturer in Pathology and Dr. (Mrs.) Ratna, M.D., Lecturer in Pathology, M.L.B.Medic College, Jhansi, who gave their valuable suggestions from time to time.

I have to words to express my gratefulness to my father Shri Ram Adhar for his uncessant inspirations durin the course of studies.

I acknowledge with deep sense of appreciation, the timely help and instigation of Dr. Y.N. Pandey to complete this work. I gratefully acknowledge the help of Dr. Suresh Singh for his valuable suggestions and honest criticisms.

I am extremely obliged to Dr. Surendra Pathwar who helped me several occasions and created a healthy atmosphere needed for my studies.

I am undoubtedly grateful to Dr. Sushil Kumar Roosia, Dr. Dilip Kumar, Dr. Surendra Pal, Dr. Abdul Moid, (D.C.P. student) for their heartly co-operation.

I am highly thankful to Mr. Ram Sanehi, Mr. Pahalwan Singh, Mr. R.C. Sachan, Mr. Shiv Lochan, Mr. B.M. Sharma, Mr. R.C. Jain, Mr. Jwala Singh, Mr. V.N. Mishra (for his excellent photography).

I thank Mr. Laxman Prasad, Mr. Ghanshyam (Sweeper), Mr. Anil Kumar and Mr. Ram Sewak who helped me a lot during this work.

I am also thankful to shopkeepers who provided me hearts of few animals.

Lastly but not the least I gratefully acknowledge the co-operation of all my friends along with Mr. Devendra Kumar and Mr. Siya Ram during completion of my thesis work.

For laborious and fair typing I am thankful to Mr. Kanhaiya Lal.

Dvijendra Nath

Dated : 10 October, 1988 (DVIJENDRA NATH)

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INTRODUCTION

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It has been noticed that animals immunized with heterologous heart tissue produce circulating anticardiac antibodies reactive with hearts of immunizing species, (Kaplan 1958a, 1958b; Gery et al 1960, 1961; Halbert et al 1968; Lin et al 1972; Chaturvedi and Gupta et al 1971; Gupta 1976, 1977). Using immune precipitation techniques of cross reactivity between heart antigens and anti heart antibodies, a number of antigenic systems have been demonstrated in the heart of different species of mammals (human, rabbit, rat, guinae pig, beaf and monkey etc).

It is apparent that cardiac antigenic systems of mostly the mammals has been studied so far, whereas the immunological response of the cardiac antigens of lower vertebrates viz. Fish, Frog, Tortoise, Hen and Bat still remain unexplained. The mammalian heart, which is complex having four chambers has been shown to have multiple antigenic systems (Lin et al 1972; Gupta 1977).

It is apparent that during the course of evolution from fish to man, the heart became from two to three and four chambered starting from fishes to amphibia and to mammals. In other words with more and more evolution, the heart has became more specialized and more complex.

However, if this complexity is also reflected in the pattern of cardiac antigenic systems contained in the heart of these animals, has not been studied so far.

In view of the lack of information concerning the properties of cardiac antigenic fractions of various species viz. Fish, Frog, Tortoise, Hen, Bat and Pig and apparent specificity of some of them for heart tissue, it was felt of paramount importance to characterize the antigenic systems contained in the heart and to determine their possible immunological relationship with the complexity adopted by the heart right from pisces upto mammalia.

In this purview the attempt has been made to study the auto-immune behaviour of cardiac antigens of various species of animals by producing anticardiac antibodies in rabbits immunized with heart antigens of Fish, Frog, Tortoise, Hen, Bat and Pig. The cross reactivity of cardiac antigens against anti-heart antiserum and the physico-chemical characterization of these antigens has also been studied. This information shall be of help in speculating the phylogenetic development of cardiac antigens, as the heart became more complex from pisces to mammalia.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The study of the cardiac antigens of various species of animals has been well documented since long time, and is based on the immunoexperimental production of autoantibodies and autodestructive lesions of heart preceded by immunization of animal with :

(a) Homologous and/or heterologous heart extracts (Cavelti 1947, Kaplan and Craig 1958, Davies et al 1964, Chaturvedi and Mehrotra 1967, Gupta 1977 & 1980).

(b) Passive transfer of humoral anticardiac antibodies (Chaturvedi and Mehrotra 1967 , Gupta 1978).

(c) And/or after passive transfer of cellular antibodies, either through intravenous injection of immune cell suspension or by transplantation of human spleens (Chaturvedi and Mehrotra, 1967).

Cavelti (1947 a,b), for the first time reported the experimental production of autoantibodies and autodestructive lesions of the heart, skeletal muscle and connective tissue in rabbits and rats after immunization of these animals (rabbits and rats) with a mixture of killed streptococci and emulsion of tissue viz. heart connective tissue and skeletal muscle. He suggested that streptococci enhance the antigenic activity of these tissues and autoantibodies are produced which cause cardiac damage.

Maekawa (1951) and Shoji (1955) demonstrated the antigenicity of lipid fraction of mammalain heart tissue and experimentally proved that antigen of heart was organ specific and induced interstitial myocarditis when injected with other carrier proteins.

Kaplan et al (1958 a & b) produced anticardiac antibodies and cardiac lesions after immunizing the rabbits with homogenates of beaf heart or rat heart incorporated in Freund's adjuvant or alumina gel as detected by immunofluorescence and complement fixation techniques. The cardiac lesions were characterized by myofibre necrosis, interstitial inflammation and rarely extensive myocardial fibrosis.

The cardiac antigen was reported to be an alcohol soluble organ specific constituent of the sarcoplasm of striated muscle and was distinct from cardiolipin as evident from serological and immunofluorescent techniques, (Kaplan, 1958c). Later on immunofluorescent, complement fixation and agglutination tests suggested the presence of atleast two separate alcohol soluble constituents of myocardial cell sarcoplasm as reactants (Kaplan, 1960).

Gery et al (1960) immunized the rabbits with homologous and heterologous heart extract (rabbit and rat heart) and successfully produced the heart specific autoantibodies in rabbits, using haemagglutination, gel precipitation

and complement fixation techniques. However, he found that neither the sera of animals had any cytopathic effect on rabbit heart tissue culture for any lesion was found in the heart of immunized animal. The dog heart extract was also included during the course of study. Gel diffusion, latex agglutination, complement fixation, and passive cutaneous anaphylaxis revealed that antibodies produced against heterologous heart were species specific and not the organ specific, while tanned red cell agglutination test showed the presence of organ specific antibodies which was confirmed by haemagglutination inhibition and neutralization tests (Gery et al, 1961a). The organ specificity of such antibodies using tanned cell haemagglutination test has been further confirmed by Ehrenfeld (1961) and Chaturvedi and Mehrotra (1967). It was also shown that heart extracts admixed with adjuvants produce high titres of haemagglutinating antibodies. And those without adjuvant were not antigenic (Gery et al, 1961b).

In 1961 Kaplan et al produced and isolated organ specific antibodies after immunizing rabbits with homogenates of heterologous heart from beaf or rat incorporated in aluminium hydroxide gel. The organ specific antibodies were detected by complement fixation test immunofluoresent technique and precipitation reaction. The cardiac lesions produced by organ specific antibodies had no correlation with anticardiac antibody titre.

These authors further reported that the antisera to heterologous heart react with antigenic material found associated with heart tissue from sarcoplasmic reticulum of myofibre and not with other organs of rabbit including skeletal muscle i.e. the antibodies produced were organ specific. In previous published reports by Henle et al (1940 and 1941), the antigenic reactivity of heart was held to be characteristic, and it was demonstrable in high speed sedimentable fraction of bovine and mouse heart. No cross reactivity between these species or with rabbit heart was observed. Indefinite or negative results were obtained in studies with skeletal muscle. Bailey and Raffel (1941) observed that within each of the species ox, dog and man heart, and skeletal muscle shared common heat stable haptens which were distinct from those of other organs. The individuality of heart and skeletal muscle haptens could not be shown.

Kaplan et al (1962) again reported that in rabbits immunized with heart homogenate of heterologous species e.g. beaf or rat formation of antibodies reactive with normal rabbit heart tissue is induced which are demonstrable by flocculation, complement frixation, immunoflourescent and haemagglutination techniques (Elson et al, 1958; Cowie et al, 1961; Kameyama et al, 1962). A sedimentable fraction of antigen of saline extract of normal rabbit heart

reactive with such antisera was shown to be present only in heart tissue and not in other organ extracts tested. Immunoflourescent studies suggested that this organ specific antigen was localized in the cardiac myofibre to sites adjacent to and between myofibrils. The possibility that immunization with heterologous heart could induce the cardiac lesions in rabbits attributable to antibodies cross reactive with autologous heart was investigated. It was observed that rabbits given repeated subcutaneous injections of heterologous heart in alumina gel adjuvant exhibited focal cardiac lesions as evident by histopathological findings.

Davies et al (1964) produced circulating anticardiac antibodies and myocardiac lesions in different experimental animals injected with homologous or heterologous preparation of heart tissue with or without Freunds adjuvant. Rabbits, rats and hamsters were immunized with homologous heart homogenate with Freund's adjuvant showed either haemagglutinating antibodies or focal myocarditis or both. Besides other experiments with other animals, the rabbits, guineapigs and rats were given heterologous heart homogenate with or without Freund's adjuvant showed production of anticardiac antibodies in almost all the cases along with same type of focal myocarditis. There was no correlation between antibody titre and myocardial lesions.

Chaturvedi and Mehrotra (1967a, 1967b, 1968); Chaturvedi and Gupta (1971); Chaturvedi et al (1973); Chaturvedi et al (1976) demonstrated the production of circulating anticardiac antibodies in rabbits after immunization of these animals with saline extract of rat heart mixed with complete Freund's adjuvant. The antibodies thus produced were organ specific as detected by modified tanned red cells agglutination technique.

Halbert et al (1968, 1970) demonstrated that the immunization of rabbits with homologous and heterologous cardiac antigens mixed with complete Freund's adjuvant resulted in the production of anticardiac antibodies which reacted with the development of multiple precipitin bands against soluble rabbit heart antigens. Rabbits immunized heterologously by using heart extracts of rat, guineapig and bovine reacted with the development of anticardiac antibodies, which gave reaction of identity against the heart extracts of immunizing species as detected by immunodiffusion techniques. It was also shown that some of the autoantibodies were directed against antigens restricted to the heart as judged by comparative immuno-diffusion tests with other rabbit tissue extract.

Holm et al (1970) demonstrated the production of cardiac autoantibodies and studied the effect of various physical agents e.g. enzymes proteinases, heat and pH. On

reactivity of autoantibodies. After exposure to three proteinases (trypsin, papepsin and papain) the reactivity was shown to be lost. Most of them were found non reactive after exposure to 50°C temperature or 60°C while the d-protein required heating above 85°C. They were reported also to differ in their sensitivity to pH change, but all because non reactive by short exposure to pH extreme (2 and 12). The cardiac autoantigens proved separable from each other by purification procedures. Numerous immunodiffusion patterns with these fractions and a more potent autoantibody concentrate, indicated that upto 7 rabbit constituents may act as autoantigens. Attempts were also made to identify the heart autoantigens with known cardiac proteins, but the rabbit antirabbit heart autoimmune precipitates were reported as failed to reveal any of the enzyme activities; no specific esterase, acid phosphatase, alkaline phosphatase, glucosaminidase, beta glucoronidase.

Chaturvedi and Gupta (1971) demonstrated the antigenicity and immunopathogenicity of monkey heart extract and its five chemical fractions viz. protein, proteinfree supernate, polysaccharide, boiling methonol resistant and lipid fractions. It was shown that cardiac lesions could be produced in albino rats by administration of total heart extract, protein, proteinfree supernate and polysaccharide fractions mixed with the complete Freund's adjuvant while no injury could be

produced with boiling methonol resistant and lipid fractions. The tanned red cell agglutinating, agar gel precipitating and cellular antibodies were produced by the heart extract, protein, proteinfree supernate and polysaccharide fractions. They showed that there are atleast two antigenic protein fractions in the heart. The polysaccharide are also antigenic but acted like hapten.

Thompson et al (1971) revealed complex cardiac autoimmune system similar to rabbits. Upto 4 cardiac autoantibodies were demonstrated by two directional immunodiffusion in some animals, several of antigens involved in these reactions were shown to be restricted to cardiac tissue, and the rat heart autoantibodies were reported to react with analogous proteins in other mammalian hearts (rabbit, human bovine, and guineapig), as was the case in rabbit system.

Lin et al (1972) studied the properties of 5 cardiac antigens in human heart which cross react with rabbit anti-rabbit heart autoantigens. All were exposed to various physical and chemical agents. All the antigens were rendered undetectable after exposure to proteinases but were unaffected by ribonulease, or desoxy ribonuclease, they were salted out with ammonium sulphate and they were shown to be variably labile to heat or pH extremes, and their elution characteristics from hydroxylappetite and sephadex G-100 were similar. It was also demonstrated that the human cardiac proteins detectable with

rabbit antirabbit heart autoantibodies were found to be organ specific. Urea with or without dithiothreitol was shown to inactivate only 2 of the human cardiac autoantigens.

Lin and Halbert (1972) demonstrated at least twelve cardiac "tissue" antigens after absorption with human plasma which was further absorbed with human kidney and skeletal muscle. It was shown that the resulting antiserum reacted only with human heart extract in immunodiffusion tests. The antigen restricted to heart was labelled as autoantigen B having a molecular weight of about 160,000 and isoelectric point of pH 5.2. Moreover the relationship of rabbit heart autoantibodies was revealed with human heart to those produced after immunization with rabbit heart, was confirmed. Lin and Halbert (1972) further demonstrated D autoantigen which was human heart autoantigen cross reactive with rabbit antirabbit heart autoantibody. This autoantigen was purified by sequential salting out with ammonium sulphate, chromatography of DEAE cellulose filtration through sephadex G-200 gel. The isoelectric point of antigen D was 5.0, appeared to show biphasic distribution and was heterogeneous in several other respects, molecular weight was about 68000, sedimentation efficient of 3.5, and diffusion coefficient of 6.68×10^{-7} cm^2 sec^{-1}, with the above work Lin et al (1971, 1972) tried to establish a potential correlation between pathological autoimmune system of human body e.g. cardiac autoimmune response in various heart diseases (Kaplan et al, 1968 and

Ellis et al, 1970) and experimental autoimmune response, because of the probability that the same proteins are involved in experimental and clinical autoimmune systems.

Chaturvedi and Davies et al (1973) attempted to purify and characterize the protein antigens related to autoimmune carditis in the heart extract of albino rats by ammonium sulphate fractionation, molecular gel filtration and polyacrylamide gel electrophoresis. Out of nine antigenic proteins of albino rat heart, three were found antigenic to rabbits and were labelled as CAI, II and III.

Gupta (1976) demonstrated the comparatively better immunological response in rabbits immunized with a mixture of total heterologous heart homogenate and complete Freund's adjuvant in equal volumes than in the rabbits immunized with a mixture of clear heart extract and complete Freund's adjuvant. Sera of the first group of animals showed two precipitin bands against monkey heart while the later showed only one precipitin band. It was suggested that some of the potent cardiac antigenic fractions may not be sufficiently soluable in saline and may occur in particulate form.

Gupta (1977) further investigated the probable chemical nature and immunization potential of saline extract of rat heart and its various chemical fractions viz.proteins, polysaccharides, and lipids. Tanned red cell agglutination and microagar gel diffusion precipitation technique were

adopted and cross reactivity was observed using agar gel diffusion precipitation. It was suggested that rat heart contains atleast 2 protein antigens and one polysaccharide hapten.

The characterization of rat heart proteins was done by Natu et al 1977 using, simple methods. The cardiac protein antigens (CA I and CA III) were obtained in totally pure form by prolonged electrophoresis of 100% ammonium sulphate saturated heart extract. Pure CA III produced maximum cardiac damage when injected with Freund's adjuvant while little damage was produced by CA I. CA III showed tanned red cell agglutination antibody titre of 128 while it was 104 in animals receiving CA I at 3 weeks. In contrast the mean titres were more marked with CA I (360) and heart extract (326) at six weeks. The migration inhibition test was also done. It was observed that CA I is good in producing humoral antibody but is week in producing cardiac lesions and cell mediated immunity (CMI).

A further study was undertaken by Natu and associates 1980 selectively to obtain CA II after selective elimination of CA I and CA III. CA III and CA I were destroyed after exposure to 85°C and 90°C temperatures respectively. CA I and CA III were found stable at acidic and basic ranges of pH while CA II was stable at neutral pH (7) only. Trypsin treatment was also studied. 10% formaldehyde solution selectively precipitates CA III and CA I leaving behind CA II in solution. The yield of CA II was only one fourth of original concentration.

MATERIAL AND METHOD

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EXPERIMENTAL ANIMALS

The study was carried out on healthy adult albino rabbits of either sex (10 males and 2 females) weighing 1.5 to 1.8 kgs. These animals were obtained from Central Animal House of this college. All the animals were maintained on standard balanced diet.

ANTIGENS

Saline extract of the heart of the following species of animals was used :

1- Fish
2- Frog
3- Tortoise
4- Hen
5- Bat
6- Pig

Saline extract of heart of each species of animals was termed as total heart extract.

PREPARATION OF TOTAL HEART EXTRACT

For removal of heart, the animals were killed using excess of ether. The heart was carefully removed aseptically. It was freed off covering fibro-fatty tissue;

cardiac chambers were opened, all the blood was drained out and the tissue was washed in several changes of sterile cold isotonic saline, so as to remove even traces of blood.

One gm of heart tissue was finely divided with scissors, washed in several changes of sterile cold isotonic saline and homogenized with 10 ml sterile isotonic saline in a tissue homogenizer at 4,000 r.p.m. for 30 minutes in cold. This homogenate was cold centrifuged at 6,000 r.p.m. for 30 minutes. The clear supernate, 10% saline extract of heart was used afresh or stored frozen in small aliquots in sterile screwcapped glass test tubes for further use.

The heart extract mixed with equal volume of complete Freund's adjuvant was used as cardiac antigen for immunization of rabbits.

FREUND'S ADJUVANT

Complete Freund's adjuvant manufactured by DIFCO Laboratories (USA) was used. Equal volume of heart extract and complete Freund's adjuvant contained in a screwcapped bottle were thawed for 20-30 minutes till the mixture converted into white viscous emulsion; which was used for the immunization of rabbits.

EXPOSURE OF HEART EXTRACT TO HEAT, pH AND AMMONIUM SULPHATE PRECIPITATION.

1. Exposure to Heat :

The 10% saline extract of heart of different species of animals was exposed to 55°C, 60°C, 70°C, 80°C and 100°C temperature for 30 minutes each. For this purpose one ml of heart extract was taken in screwcapped tubes. Tube number one was incubated at 55°C for 30 minutes in a water bath; subsequent tubes were similarly incubated at temperatures 60°C, 70°C, 80°C and 100°C for 30 minutes each. The heat exposed heart extract was cold centrifuged at 4,000 r.p.m. for 20 minutes and supernate was stored frozen for use in subsequent experiments.

2. Exposure to pH :

Saline heart extract of different animals was exposed to variable pH for a specified time. For this purpose 1 ml aliquots of heart extract were exposed to pH 4.5, 6, 8 and 9.5 for one hour at room temperature. The required pH was adjusted with N/10 Hcl or N/10 NaoH and cleared by cold centrifugation at 3,000 r.p.m. for 30 minutes.

3. Salting out with Ammonium Sulphate :

Freshly prepared 10% saline heart extract of each animal species was variously viz 20%, 40%, 60%, 80% and 100% saturated with ammonium sulphate. For this purpose previously calculated amount of ammonium sulphate (crystals) was gradually added to one ml of heart extract and the contents were constantly, stirred for few minutes. The precipitate was allowed to stand over might at 4°C in a refrigerator. Next morning the contents were centrifuged at 4,000 r.p.m. for 20 minutes in a cold centrifuge. The clear supernate was pipetted off, the protein precipitate was suspended in 1 ml cold sterile isotonic saline and dialized against distilled water, so as to remove even traces of ammonium sulphate ions. To ensure the complete removal of ammonium sulphate, small aliquots were drawn from time to time from dylysing media fluid (distilled water) and tested with barium chloride for the presence of sulphate ions. The sulphate ions free protein precipitate thus obtained was reconstituted with normal saline to the original volume, and used as specified protein fraction of antigen for further study.

PREPARATION OF ANTICARDIAC ANTISERA

The anticardiac antisera were raised against total heart extract of various animal species viz. Fish, Frog, Tortoise, Hen, Bat and Pig, after immunization of rabbits by subcutaneous route and also by intravenous route as per immunization schedule described below.

Immunization of animals

The immunization of rabbits was performed by injecting cardiac antigen (emulsion of heart extract and complete Freund's adjuvant in abdominal flanks, subcutaneously in doses of 0.2 ml once a week for four weeks. It was followed by intrabenous injection into the marginal ear vein of 0.2 ml of saline heart extract once a week consecutively for next 2 weeks. The schedule of immunization of rabbits is shown in table 1 in detail.

Table - 1 : Schedule for immunization of rabbits with cardiac antigen.

Rabbit No.	Immunizing cardiac antigen	Route of administration of cardiac antigen at different intervals (weeks)					
		1wk	2wk	3wk	4wk	5wk	6wk
1-	Fish	0.2 ml subcutaneous injection of cardiac antigen at weekly interval for four weeks.				0.2 ml IVI of saline heart extract at weekly intervals for 2 weeks	
2-	Fish						
3-	Frog	Same as above				Same as above	
4-	Frog						
5-	Tortoise	Same as above				Same as above	
6-	Tortoise						
7-	Hen	Same as above				Same as above	
8-	Hen						
9-	Bat	Same as above				Same as above	
10-	Bat						
11-	Pig	Same as above				Same as above	
12-	Pig						

Bleeding of rabbits was done from marginal ear vein and 4 to 5 ml of blood was obtained from each rabbits; serum

was separated by cold centrifugation at 4,000 r.p.m. for 20 minutes and stored frozen till used.

DETECTION OF ANTICARDIAC ANTIBODIES :

The anticardiac antibodies were detected in these sera using micro-agar gel diffusion, immuno-electrophoresis and immune precipitation electrophoresis techniques.

MICRO AGAR GEL DIFFUSION TECHNIQUE :

Micro modification of Outcherlony's double gel diffusion technique as described by Grasset et al (1958) was used for detection of circulating anticardiac antibodies in the sera of experimental animals. Microscopic glass slides (75x25 mm) were evenly covered by 3 ml of buffered agarose gel.

Preparation of Sodium Barbital Acetate Buffer (pH 8.4)

The buffer of following composition was used :-

- Sodium diethyl barbiturate ($C_8 H_{11} N_2 Na. 3 H_2O$) - 8.142 gm
- Sodium acetate ($CH_3 COONa. 3 H_2O$) - 6.476 gm
- Hydrochloric acid (Hcl) 0.1 N - 90 ml
- Distilled water - 1000 ml
- pH - 8.4

The buffer was always used afresh.

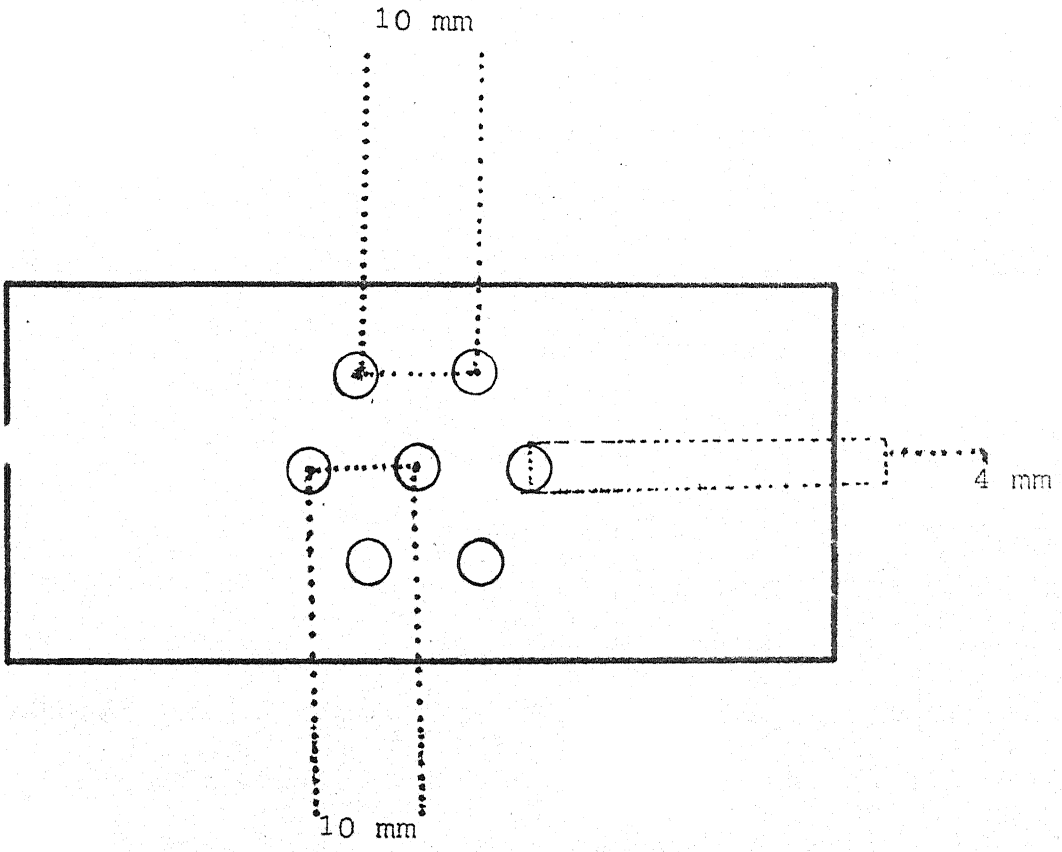


Fig 1 : Agar gel pattern used for AGDP. The wells were 4 mm in diameter and the centres of two adjoining wells were 10mm apart; the central well O contained anticardiac antiserum and the peripheral wells O had different cardiac antigens.

Preparation of 1% buffered agarose

Buffered agarose (1%) was prepared by dissolving 1 gm agarose (Difco) in 100 ml buffer solution. For this purpose the contents were thoroughly shaken in a conical flask and kept in boiling water bath with intermittent shaking for 30 minutes or till a clear transparent solution was obtained. It was used afresh or stored in 25 ml aliquots at 4°C in a refrigerator. Just before use the agarose was melted by keeping the tube in boiling water bath for 10 minutes. Prewarmed microscopic glass slides (75x25 mm) were evenly covered with 3 ml of buffered agarose and the gel was allowed to settle at room temperature. Wells of different pattern (according to need) were made in the agar using a predesigned card template (Fig 1 & 2) with the help of a cork boarer. The distance between the adjacent two wells was always kept contant and the centres of the adjoining two wells were 1 cm apart, and the lateral distance between two adjacent wells was also kept constant.

The moisture from the surface of agar slides so prepared was removed after incubation at 37°C for 10 to 15 minutes in an incubator. Now the wells were charged with antisera and antigen to be tested. These slides were incubated at °C for 48 hours in moist atmosphere. The slides were examined for presence of precipitin lines under oblique illumination.

It was observed that whatever lines had to develope they appeared within 24 hours and reached their maximum intensity within 48 hours. Some times the precipitin lines developed as early as 6 to 8 hours. Beyond 48 hours there was neither an increase in the density or sharpness; nor the formation of new lines and infact further incubation sometimes caused broadening and fainting of precipitin lines. The number of precipitin lines developed were noted and recorded.

Washing and drying of slides

Unprecipipated protein was washed off by several change of cold sterile isotonic saline over a period of 48 to 72 hours at $4^{o}C$ temperature in a refrigerator. Thereafter the slides were carefully covered with wet whattman filter paper No.1 and allowed to dry at room temperature. Care was taken to remove any air bubbles between filter paper and the layer of agarose so as to avoid the development of cracks in the agarose. When dry the filter paper detached by itself. Thereafter the precipitin bands were stained using Coomassie brilliant blue.

Staining of slides

Coomassie brilliant blue solution of following composition was used :-

omassie Brilliant Blue	-	0.5 gm
96% Methanol	-	45.0 ml
Glacial acetic acid	-	10.0 ml
Distilled water	-	45.0 ml

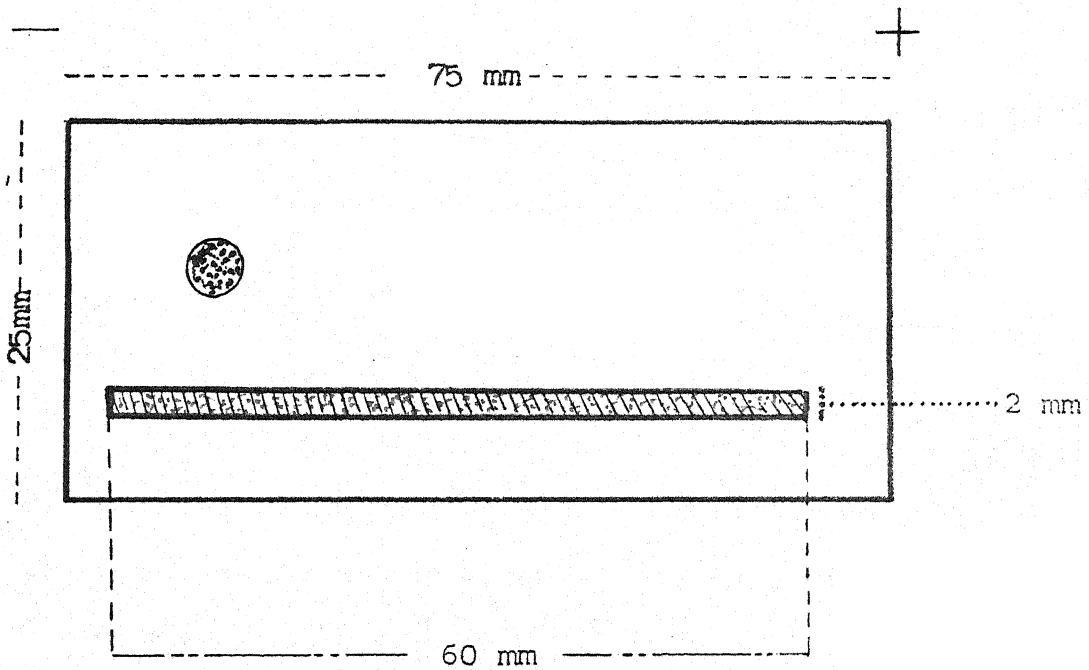


Fig 2 : Agar gel pattern used for immunoelectrophoresis (IEP). The well was charged with saline heart extract and subjected to constant current electrophoresis for 2 hours; 60 x 2 mm sloght was cut in the agar and charged with anticardiac antiserum.

The staining was done for 10 minutes and the excess of stain was decanted and the precipitin lines were differentiated by several washings for 10 minutes each in acetic acid methanol solution of following composition :-

Glacial acetic acid	-	50 ml
Methanol	-	225 ml
Distilled water	-	225 ml

The slides were cleared and dried in air at room temperature and stored for permanent record.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis procedure of Wadsworth and Hanson (1960) as described by Gupta (1978) was adopted using barbital buffer pH 8.4. A 4mm hole was punched in the agarose, it was charged with a particular cardiac antigen to be tested and subjected to electrophoresis for 2 hours at a constant current of 2 m.A per slide. The circuit was completed by connecting the ends of agarose covered slides with buffer in the electrophoresis chamber using 2.5 cm broad strips of wet whattmen No.3 filter paper. Throughout the duration of electrophoretic run, the chamber was kept in refrigerator, and connected outside to power supply unit, so as to avoid any denaturation of proteins due to extreemes of heat generated during electrophoresis. After the completion of electrophoretic run a slot 2 mm in width and 60 mm in length was cut 1 cm away from the well (Fig 2). In this slot corresponding anticardiac antiserum was placed and slides were

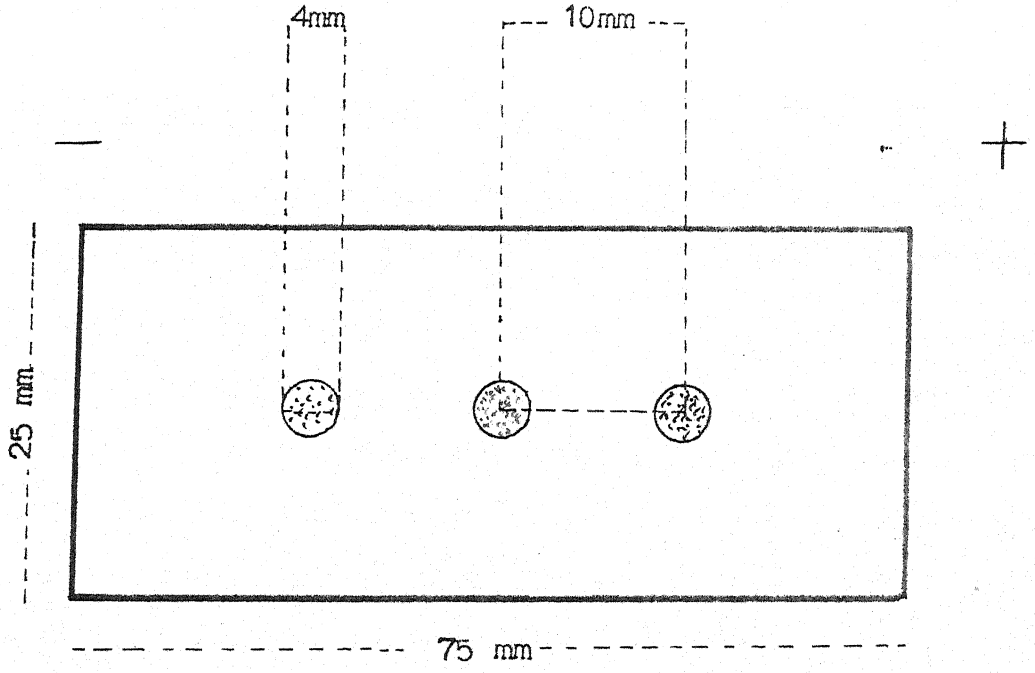


Fig 3 : Agar gel pattern used for immune precipitation electrophoresis. The wells were 4mm in diameter and the centres of two adjoining wells were 10mm apart. The constant current electrophoresis was performed for 2 hours. The antigen was contained in ● wells and the anticardiac antiserum in ○ wells.

incubated at 4°C for 24 to 48 hours in a refrigerator and examined for the development of any precipitin lines under oblique illumination. The precipitin patterns were recorded and thereafter the slides were washed, dried and stained as already described.

IMMUNE PRECIPITATION ELECTROPHORESIS TECHNIQUE

Immune precipitation electrophoresis, as described by Psendorfer et al (1970) with suitable modifications was also used for detection of circulating anticardiac antibodies. Buffered agarose (1%) pH 8.4 was used. Wells 4.0 mm in diameter were punched using a predesigned card board template (Fig 3), the distance between two adjacent wells was 10 mm. The central well of each set was charged with heart extract or its variously treated components while the two adjacent wells were charged with anticardiac antiserum. The electrophoresis was performed using a current of 2 mA per slide ; the other conditions for electrophoresis were same as describe for immunoelectrophoresis. The precipitin lines were recorded under oblique illumination after completion of electrophoretic run. Thereafter the slides were washed, dried and stained by method as already described.

OBSERVATIONS AND RESULTS

OBSERVATIONS AND RESULTS

ANTICARDIAC ANTIBODIES PRODUCED AFTER IMMUNIZATION WITH HETEROLOGOUS HEART ANTIGEN :

All the rabbits immunized with heart antigens of Fish, Frog, Tortoise, Hen, Bat and Pig reacted with the development of anticardiac antibodies as detected by agar gel diffusion (A.G.D.P.), Immunoelectrophoresis (I.E.P.) and immune-precipitation electrophoresis (I.P.E.) techniques. The findings of the cross-reactivity of anticardiac antiserum with corresponding heart extract of different species of animals have been shown in detail in table 2.

As evident from this table, fish and tortoise heart extracts reacted with anti-fish-heart antiserum and anti-tortoise heart antiserum respectively with the development of two precipitin bands in agar gel diffusion, immuno-electrophoresis and immuneprecipitation electrophoresis techniques, thereby indicating that saline extract of fish and tortoise hearts contain atleast two soluble antigenic fractions.

Cross-reactivity of frog heart extract, hen heart extract and the bat heart extract against corresponding anti-cardiac antiserum revealed the presence of 3 precipitin bands by all immunodiffusion techniques viz - AGDP, IEP and IPE, indicating thereby that the saline extract of the heart of these species of animals contained atleast three soluble antigenic fractions.

Table - 2 : Cross-reactivity of different anticardiac antisera against heart extracts of various species of animals.

Rabbit No.	Anticardiac antiserum against heart extract of	Testing antigen	Number of precipitin systems		
			AGDP	IEP	IPE
1 & 2	Fish	Fish heart extract	2	2	2
3 & 4	Frog	Frog heart extract	3	3	3
5 & 6	Tortoise	Tortoise heart extract	2	2	2
7 & 8	Hen	Hen heart extract	3	3	3
9 & 10	Bat	Bat heart extract	3	3	3
11 & 12	Pig	Pig heart extract	4/5	4/5	4/5

AGDP = Agar gel diffusion precipitation.

IEP = Immunoelectrophoresis.

IPE = Immunoprecipitation electrophoresis.

Immunization of rabbits against pig heart antigen resulted in the development of anticardiac antibodies which reacted with corresponding heart extract with development of atleast 4 distinct and one faint precipitin lines by AGDP technique, 4 to 5 precipitin lines were also observed after immunoelectrophoresis and immune-precipitation electrophoresis procedures.

These observations indicate that the frog, tortoise, hen and bat heart antigens are more complex than the fish heart antigen; and the pig heart antigens are the most complex as evident by the presence of largest number of precipitin systems demonstrable in pig heart extract reacting with anti-pig heart antiserum.

The comparative evaluation of AGDP, IEP and IPE techniques for the detection of anticardiac antibodies in the sera of animals immunized with a particular heart antigen revealed that the sensitivity of all the 3 techniques viz. AGDP, IEP and IPE for the detection of anticardiac antibodies/ cardiac antigens was almost similar.

The cross-reactivity of different anticardiac antisera against the heart extract of various species of animals is shown in table 3. Since AGDP, IEP and IPE techniques were found almost equally sensitive for the detection of circulating anticardiac antibodies/cardiac antigens, for this experiment only AGDP technique was used.

Table - 3 : Cross-reactivity of different anticardiac antisera against heart extracts of various species of animals.

Anticardiac anti-serum against heart of	Testing cardiac antigen (AGDP technique)					
	Fish	Frog	Tortoise	Hen	Bat	Pig
Fish	+	(-)	(-)	(-)	(-)	(-)
Frog	(-)	+	(-)	(-)	(-)	(-)
Tortoise	(-)	(-)	+	(-)	(-)	(-)
Hen	(-)	(-)	(-)	+	(-)	(-)
Bat	(-)	(-)	(-)	(-)	+	(-)
Pig	(-)	(-)	(-)	(-)	(-)	+

AGDP = Agar gel diffusion precipitation.

\+ = Reactivity with corresponding antiserum.

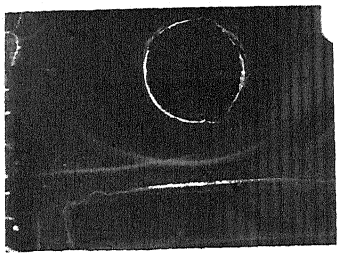
(-) = No cross reactivity.

As evident from this table the anticardiac antiserum against the cardiac antigen of a particular species of animal reacted with the heart extract of only immunizing species of animal and not with the heart extract of any other animal. It would suggest that anti-cardiac antibodies evoked against the heart extract of different species of animals, under study, are species specific.

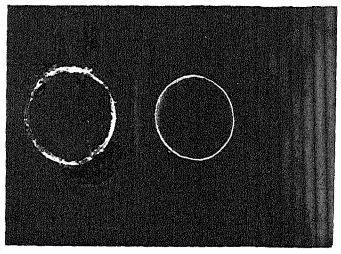
EFFECT OF HEAT ON CARDIAC ANTIGENS :

The effect of heat on heart antigens was studied by exposure of saline heart extract of different species of animals to varying temperature.

It was observed that the fish heart extract exposed to a temperature of 55°C and 60°C for 30 minutes reacted with corresponding anti-fish-heart antiserum to produce a single precipitin band. No precipitin band was obtained on cross-reactivity of anti-fish-heart antiserum against aliquots of fish heart extract exposed to temperatures of 70°C, 80°C and 100°C for 30 minutes. These findings are shown in detail in table 4.



Two precipitin bands demonstrated by immuno-electrophoresis (IEP technique) obtained after reaction of fish heart antigen with anti-fish-heart antiserum.



Single precipitin band in immuno precipitation electrophoresis (IPE technique) obtained after anti-fish-heart antiserum and reactivity of fish heart antigen exposed at a temperature of 55°C for 30 min; one of the fish heart antigen was susceptible to this temperature.

Table - 4 : Effect of heat on fish heart antigens.

Technique adopted	Number of precipitin bands after exposure of fish heart extract to different temperatures					
	UEF	55°C	60°C	70°C	80°C	100°C
AGDP	2	1	1	0	0	0
IEP	2	1	1	0	0	0
IPE	2	1	1	0	0	0

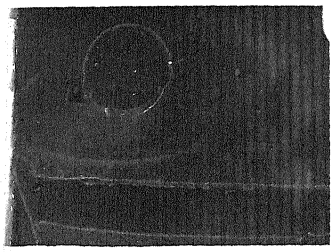
UEF = Fish heart extract unexposed (not exposed to heat).

0 = No precipitin band observed.

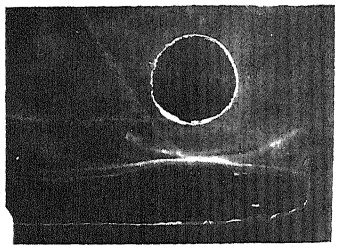
The results of this experiment indicate that one out of two antigens contained in the fish heart is highly heat labile and is destroyed after exposure even to 55°C for 30 minutes, as evident by the fact that unexposed fish heart extract reacted with the development of two precipitin bands, whereas the aliquots of fish heart antigen exposed to temperature of 55°C and 60°C for 30 minutes showed only one precipitin band against anti-fish-heart antiserum.

However, both the antigenic fractions contained in fish heart extractwere destroyed at temperature of 80°C or more, as evident by the absence of any precipitin bands in cross reactivity experiments using anti-fish-heart antiserum and aliquots of fish heart extract exposed to 80°C and 100°C temperatures for 30 minutes.

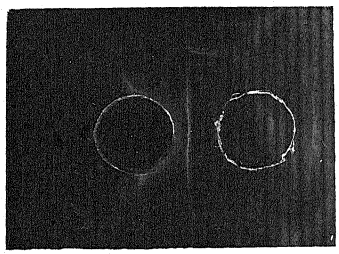
The frog heart extract exposed to varying temperature, on cross reactivity with corresponding anti-fish-heart serum showed that the frog heart extract exposed to temperature of 55°C for 30 minutes reacted with the development of one precipitin band in AGDP and 2 precipitin bands in IPE and IEP techniques. The aliquots of frog heart extract exposed to 60°C and 70°C temperatures for 30 minutes each, reacted with the development of single precipitin line, against corresponding anti-frog-heart antiserum in AGDP, IEP and IPE techniques, whereas no precipitin bands were observed on cross reactivity of antigrog heart antiserum against aliquots of frog heart extract exposed to 80°C and 100°C. These findings are shown in detail in table 5.



Three precipitin bands obtained after reactivity of antifrog-heart antiserum with frog heart antigen (IEP technique)



Two precipitin bands obtained after reaction of anti-frog-heart antiserum with frog heart proteins obtained at 60% saturation with ammonium sulphate (IEP technique)



Single precipitin band obtained after reaction of anti-frog-heart antiserum with frog heart antigen exposed to 70°C temperature (IEP technique)

Table - 5 : Effect of heat on frog heart antigens.

Technique adopted	Number of precipitin bands after exposure of frog heart extract to different temperatures					
	UEFr.	55°C	60°C	70°C	80°C	100°C
AGDP	3	1	1	1	0	0
IEP	3	2	1	1	0	0
IPE	3	2	1	1	0	0

UEFr. = Frog heart extract unexposed (not exposed to heat).

0 = No precipitin band observed.

The results of this experiment revealed that out of three antigenic systems contained in the frog heart antigen one is highly heat labile and is destroyed even at 55°C within 30 minutes, as evident by the fact that unexposed frog heart extract reacted with the development of three precipitin bands, whereas aliquots of frog heart extract exposed to temperature of 55°C for 30 minutes resulted in the development of only two precipitin bands against anti-frog-heart antiserum. The other antigenic component of frog heart extract could withstand a temperature of 70°C for 30 minutes. However, all the three antigenic fractions present in frog heart extract were destroyed at temperature of 80°C or more, as evident by absence of any precipitin bands in cross reactivity experiments using anti-frog-heart antiserum

and aliquots of frog heart extract exposed to 80°C and 100°C temperature for 30 minutes.

The tortoise heart extract exposed to different temperatures revealed that after exposure to even 55°C for 30 minutes, tortoise heart extract did not reveal any reactivity with the corresponding anti-tortoise-heart anti-serum as evident by the absence of precipitin band by all the techniques, viz. AGDP, IEP and IPE. The findings are shown in detail in Table 6.

Table - 6 : Effect of heat on tortoise heart antigens.

Technique adopted	Number of precipitin bands after exposure of tortoise heart extract to different temperatures					
	UET	55°C	60°C	70°C	80°C	100°C
AGDP	2	0	0	0	0	0
IEP	2	0	0	0	0	0
IPE	2	0	0	0	0	0

UET = Tortoise heart extract unexposed (not exposed to heat).

0 = No precipitin band observed.

These results show that all the antigenic components contained in tortoise heart antigen are highly labile to heat and are destroyed completely after exposure to even 55°C for 30 minutes, as evident by the fact that unexposed tortoise heart extract reacted with the development of two precipitin

bands, whereas the aliquots of tortoise heart extract exposed to temperature of 55°C for 30 minutes showed no precipitin band against anti-tortoise-heart antiserum.

The hen heart extract when exposed to various degrees of temperature viz. 55°C, 60°C, 70°C and 80°C for 30 minutes, reacted with the corresponding anti-heart antiserum to produce 2, 1, 1, and 1 precipitin bands respectively, and the aliquots of hen heart extract after exposure to even 100°C temperature for 30 minutes reacted with the development of a single but faint precipitin band against corresponding anti-hen-heart antiserum in AGDP, IEP and IPE techniques. These findings are shown in detail in table 7.

Table - 7 : Effect of heat on hen heart antigens.

Technique adopted	Number of precipitin bands after exposure of hen heart extract to different temperatures					
	UEH	55°C	60°C	70°C	80°C	100°C
AGDP	3	2	1	1	1	1*
IEP	3	2	1	1	1	1*
IPE	3	2	1	1	1	1*

UEH = Hen heart extract unexposed(not exposed to heat).

* = Shows faint precipitin line.

The results of this experiment revealed that one out of three antigens present in hen heart extract is highly heat labile and is destroyed even at 55°C within 30 minutes, as evident by the fact that unexposed hen heart extract reacted with the development three precipitin bands, whereas the aliquots of hen heart extract exposed to temperature of 55°C for 30 minutes, showed only two precipitin bands against anti-hen-heart antiserum. Other two antigenic components contained in hen heart extract resisted the temperature of 55°C, but out of these two, one was destroyed after exposure to 60°C for 30 minutes. The third antigenic fraction could withstand temperatures of 70°C and 80°C for 30 minutes, and its partial antigenicity was retained even after exposure to 100°C, as evident by the development of a faint precipitin band in cross reactivity experiments using anti-hen-heart antiserum and aliquot of hen heart extract exposed to 100°C for 30 minutes.

The bat heart extract exposed to temperature of 55°C for 30 minutes, reacted with corresponding anti-bat-heart antiserum to produce only two precipitin bands. Aliquots of bat heart extract after exposure to 60°C for 30 minutes reacted with the development of a single precipitin band against corresponding anti-bat-heart antiserum in AGDP, IEP and IPE techniques, whereas no precipitin bands were observed

on cross reactivity of antibat heart antiserum against aliquots of bat heart extract exposed to 70°C or more for 30 minutes. These findings are shown in detail in table 8.

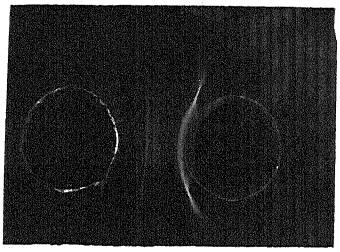
Table - 8 : Effect of heat on bat heart antigens.

Technique adopted	Number of precipitin bands after exposure of bat heart extract to different temperatures					
	UEB	55°C	60°C	70°C	80°C	100°C
AGDP	3	2	1	0	0	0
IEP	3	2	1	0	0	0
IPE	3	2	1	0	0	0

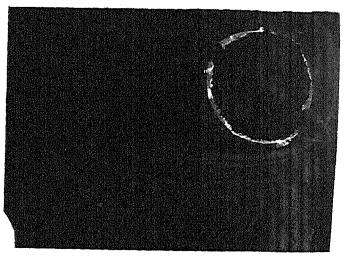
UEB = The bat heart extract unexposed(not exposed to heat).

0 = No precipitin bands.

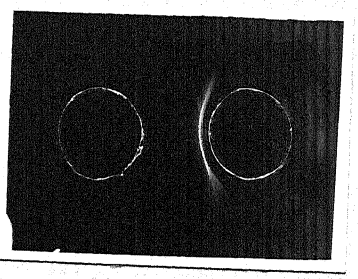
Results of this experiment show that one out of three antigenic components contained in bat heart extract is highly heat labile and is destroyed even at 55°C with in 30 minutes. As evident from the table 8, unexposed bat heart extract reacted with the development of three precipitin bands, whereas the aliquots of bat heart extract exposed to temperature of 55°C showed only two precipitin bands against anti-bat-heart antiserum. Out of these two antigenic components one was lost at 60°C as shown by the presence of only single precipitin band in the aliquots of bat heart extract



Reactivity of pig heart antigen against anti-pig-heart antiserum; five precipitin bands were obtained (IPE technique).



Reactivity of pig heart antigen against anti-pig-heart antiserum; four precipitin bands were obtained (IEP technique).



Reactivity of anti-pig-heart antiserum with pig heart antigen exposed to 60°C for 30 min; only two precipitin bands were observed, indicating that other antigenic fractions were heat labile (IPE technique).

exposed at 60°C for 30 minutes. The third antigenic component was destroyed after exposure to 70°C for 30 minutes. Thus the bat heart extract exposed to 80°C for 30 minutes, lost all the antigenicity as evident by the absence of any precipitin band in cross reactivity experiments using anti-bat-heart antiserum and aliquots of bat heart extract exposed to 80°C and 100°C for 30 minutes.

The pig heart extract exposed to varying temperature showed that the pig heart extract exposed to 55°C for 30 minutes reacted with corresponding anti-pig-heart antiserum to produce four precipitin bands by various immunoprecipitate techniques. Aliquots of pig heart extract exposed to 60°C and 70°C for 30 minutes, reacted with the development of only 2 precipitin bands, whereas the aliquots of pig heart extract exposed to 80°C and 100°C each reacted with the development of single precipitin band against corresponding anti-pig-heart antiserum in AGDP, IEP and IPE techniques. These findings are shown in detail in table 9.

Table-9 : Effect of heat on pig heart extract.

Technique adopted	Number of precipitin bands after exposure of pig heart extract to different temperatures					
	UEP	55°C	60°C	70°C	80°C	100°C
AGDP	4/5	4	2	2	1	1
IEP	4/5	4	2	2	1	1
IPE	4/5	4	2	2	1	1

UEP = Pig heart extract unexposed (not exposed to heat).

The results of this experiment show that at least one antigenic component of pig heart is extremely heat labile and is lost even at 55°C within 30 minutes. The aliquots of pig heart extract after exposure to 60°C and 70°C for 30 minutes showed two precipitin bands against anti-pig-heart antiserum, which indicates that 2 to 3 antigenic fractions present in pig heart extract are destroyed at this temperature. The aliquots exposed at 80°C and 100°C reacted feebly with the development of only 1 precipitin band against, anti-pig-heart antiserum. It is thus clear from this experiment that at least one antigenic fraction of pig heart extract could withstand a temperature of even 100°C for 30 minutes.

EFFECT OF pH ON CARDIAC ANTIGENS

The aliquots of heart extracts of various species of animals viz. Fish, Frog, Tortoise, Hen, Bat and Pig were adjusted to varying pH viz. 4.5, 6, 8 and 9.5 by the method described earlier. The cross reactivity of a particular anti-cardiac antiserum against aliquots of heart extracts at varying pH was studied using various immuno-diffusion techniques viz. Agar gel diffusion precipitation (AGDP) immunoelectrophoresis (IEP) and Immunoprecipitation electrophoresis technique (IPE).

It was observed that the fish heart extract at pH 4.5 reacted with the corresponding anti-fish-heart antiserum to produce only single precipitin band, whereas at pH 6, 8 and 9.5 it reacted with the development of two precipitin bands. These findings are shown in table 10.

Table - 10 : Effect of pH on fish heart antigens.

chnique adopted	Precipitin bands at varying pH				
	UPF	4.5	6	8	9.5
AGDP	2	1	2	2	2
IEP	2	1	2	2	2
IPE	2	1	2	2	2

The results of this experiment thus suggest that 1 out of two antigenic systems present in fish heart is highly susceptible to low pH and is destroyed at pH 4.5 as indicated by only single precipitin band against anti-fish-heart antiserum. The fish heart extract is opti mally active at varying pH viz. 6, 8 and 9.5 as evident by presence of equal member of precipitin bands obtainable after cross reacting the aliquot of fish heart extract at normal pH 7.2 against corresponding anti-fish-heart antiserum by all the 3 techniques (AGDP, IEP and IPE).

The frog heart extract exposed to pH 4.5 and 6 reacted with corresponding anti-fish-heart antiserum to produce two precipitin bands, whereas three precipitin bands were obtained on cross reactivity of anti-fish-heart antiserum against aliquots of fish heart extract at pH 8 and 9.5. These findings are shown in table 11.

Table - 11 : Effect of pH on frog heart antigens.

Technique adopted	Precipitin bands at varying pH				
	UP Fr.	4.5	6	8	9.5
AGDP	3	2	2	3	3
IEP	3	2	2	3	3
IPE	3	2	2	3	3

Results of this experiment indicate that one out of 3 antigenic systems contained in frog heart is labile at low pH as is evident by presence of only two precipitin bands at pH 4.5 and 6 against anti-frog-heart antiserum. However, the aliquots of frog heart extract exposed to pH 8 and 9.5 were not affected in anyway as indicated by presence of equal number of precipitin bands obtained after reacting the normal (pH 7.2) frog heart extract against corresponding anti-frog-heart antiserum.

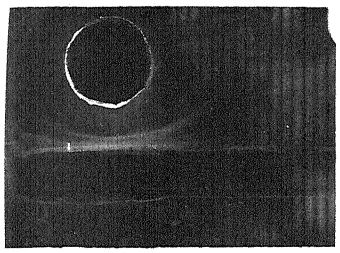
The tortoise heart extract at pH 4.5 and 9.5 did not react with the corresponding anti-tortoise-heart antiserum, whereas the aliquots of tortoise heart extract at pH 6 and 8 reacted with the corresponding anti-tortoise-heart antiserum to produce only one precipitin band in AGDP, IEP and IPE techniques. The detailed findings of this experiment are shown in table 12.

Table - 12 : Effect of pH on tortoise heart antigens.

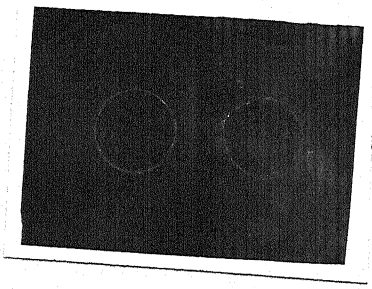
Technique adopted	Precipitin bands at varying pH				
	UPT	4.5	6	8	9.5
AGDP	2	0	1	1	0
IEP	2	0	1	1	0
IPE	2	0	1	1	0

UPT = Tortoise heart extract pH 7.2

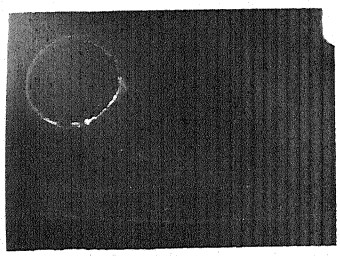
The results of this experiment revealed that both the antigenic components present in tortoise heart are highly labile at pH 4.5 as well as at pH 9.5 as evident by the absence of any precipitin line in AGDP, IEP and IPE techniques. The aliquots of tortoise heart extract exposed to pH 6 and 8 showed only 1 precipitin band against anti-tortoise-heart antiserum meaning thereby that one antigenic component of tortoise heart is highly susceptible even to little variation in pH.



Three precipitin bands obtained after reactivity of hen-heart antigen with anti-hen-heart antiserum (IEP technique).



Single precipitin band obtained after reactivity of anti-hen-heart antiserum with hen heart antigen exposed to pH 8.0 for 30 min (IPE technique).



Faint single precipitin band obtained after reactivity of anti-hen-heart anti serum with hen heart antigen exposed to $100^{o}C$ temperature for 30 min (IEP technique).

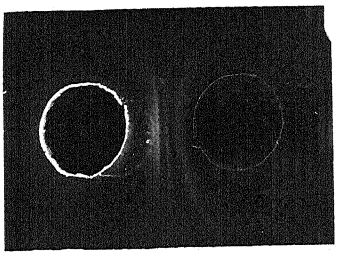
The hen heart extract exposed to pH 4.5 and 8 showed only single precipitin band against corresponding anti-hen-heart antiserum. The aliquots of hen heart extract exposed to pH 6 reacted with the development of two precipitin bands against corresponding anti heart antiserum. The aliquots of hen heart extract exposed to pH 9.5 completely lost its reactivity, as no precipitin band was observed against anti-hen-heart antiserum, whereas the aliquots of hen heart extract at pH 7.2 reacted with the development of 3 precipitin bands against corresponding anti-hen-heart antiserum. These finding are shown in table 13.

Table - 13 : Effect of pH on hen heart antigens.

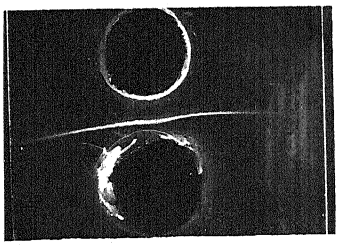
Technique adopted	Precipitin bands at varying pH				
	UPH	4.5	6	8	9.5
AGDP	3	1	2	1	0
IEP	3	1	2	1	0
IPE	3	1	2	1	0

UPH = Hen heart extract pH 7.2

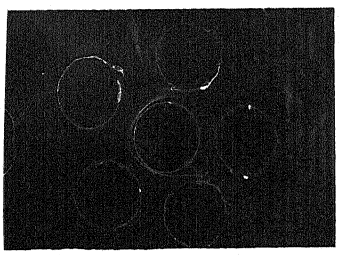
The results of this experiment indicate that out or three antigenic systems present in hen heart, 2 were destroyed at pH 4.5 and 8, while only one antigenic component was found labile to pH 6 as evident by the development of two precipitin bands in AGDP, IEP and IPE techniques. The reactivity



Three precipitin bands obtained after cross reactivity of anti-bat-heart antiserum with bat heart antigen (IPE technique)



Single precipitin band obtained after cross reactivity of anti-bat- heart antiserum with bat heart antigen exposed to 60°C for 30 min (IPE technique); indicating that other two antigenic fraction of bat heart were thermolabile.



Reactivity of anti-bat-heart antiserum with bat heart antigen at different pH (4.5, 6, 7.2, 8 and 9.5) in AGDP technique.

of hen heart extract aliquots at pH 9.5 was completely destroyed as no precipitin band was obtained in cross reactivity experiments using anti-hen-heart antiserum and aliquots of hen heart extract exposed to pH 9.5.

The bat heart extract at pH 4.5, 6 and 9.5 reacted with the development of only 1 precipitin band against corresponding anti-bat-heart antiserum, whereas the aliquots of bat heart extract exposed to pH 8 reacted against antibat heart antiserum with the development of 2 precipitin bands. The bat heart extract at pH 7.2 revealed with the development of 3 precipitin bands against corresponding anti-bat-heart antiserum in AGDP, IEP and IPE techniques. These findings are shown in table 14.

Table - 14 : Effect of pH on bat heart antigens.

Technique adopted	Precipitin bands at varying pH				
	UPB	4.5	6	8	9.5
AGDP	3	1	1	2	1
IEP	3	1	1	2	1
IPE	3	1	1	2	1

UPB = Bat heart extract pH 7.2

The results of this experiment show that out of three antigenic systems of bat heart, 2 were completely destroyed at pH 4.5, 8 and 9.5 as evident by the development

of only one precipitin line against corresponding anti-bat-heart antiserum. The bat heart antigen were found comparatively more resistant to pH 8 as evident by the presence of 2 precipitin bands after exposure to pH 8, in AGDP, IEP and IPE techniques.

The pig heart extract at pH 4.5 reacted with the development of only one precipitin band against corresponding anti-pig-heart antiserum. The aliquots of pig heart extract at pH 6 also reacted with the development of single precipitin line against corresponding antipig heart antiserum. The aliquots of pig heart extract at pH 8 reacted with the development of two precipitin bands against corresponding anti-pig-heart antiserum, however no precipitin band was observed with aliquots exposed at pH 9.5. Unexposed pig heart extract (pH 7.2) reacted with the development of 4 to 5 precipitin bands against corresponding anti-pig-heart antiserum. These findings are shown in table 15.

Table - 15 : Effect of pH on pig heart antigens.

Technique adopted	Precipitin bands at varying pH				
	UPP	4.5	6	8	9.5
AGDP	4/5	1	1	2	0
IEP	4/5	1	1	2	0
IPE	4/5	1	1	2	0

UPP = Pig heart extract pH 7.2

It was thus apparant that three to four of the antigenic components present in pig heart are labile to pH 4.5 and 6, as the pig heart extract at pH 4.5 and 6 showed only one precipitin band against corresponding anti-pig-heart antiserum. The aliquots of pig heart extraxt exposed to pH 8, showed only two precipitin bands against corresponding anti-pig-heart antiserum, whereas the pig heart extract at pH 9.5 did not show any reactivity against corresponding anti-pig-heart antiserum. It is apparent from the above results that two to three out of 4/5 antigenic systems of pig heart are specifically susceptible even to little variation in pH, and are destroyed even with slight change in pH.

SALTING OUT OF CARDIAC ANTIGENS WITH AMMONIUM SULPHATE $(NH_4)_2\ SO_4$.

The heart extract of different species of animals was variously viz. 20%, 40%, 60%, 80% and 100% saturated with ammonium sulphate and the cross reactivity of protein fractions obtained after differential precipitation by ammonium sulphate, was studied against corresponding anti heart antiserum by AGDP, IPE and IEP techniques.

Protein fraction of fish heart extract obtained at 20% saturation with ammonium sulphate, revealed no precipitin

band against anti-fish-heart antiserum. The protein fraction of fish heart precipitated at 40% and 60% saturation by ammonium sulphate reacted with the development of one precipitin band, and the protein fraction of fish heart extract precipitated at 80% and 100% saturation of ammonium sulphate reacted with development of 2 precipitin bands in each case. Fish heart extract not treated with ammonium sulphate also showed the development of 2 precipitin bands in AGDP, IEP and IPE techniques. These findings are shown in table 16.

Table - 16 : Effect of salting out with ammonium sulphate on cardiac antigens of fish heart extract.

Technique adopted	Number of precipitin bands against various protein fractions of fish heart extract obtained after salting out with different saturations of ammonium sulphate.					
	USF	20%	40%	60%	80%	100%
AGDP	2	0	1	1	2	2
IEP	2	0	1	1	2	2
IPE	2	0	1	1	2	2

USF = Fish heart extract not treated with ammonium sulphate.

The results of this experiment indicate that the protein fraction of fish heart extract precipitated at 20% saturation with ammonium sulphate was not antigenic as it failed to reveal any precipitin line against corresponding

anti-fish-heart antiserum. However, at 40% and 60% saturation, the fish heart protein fraction contained only one antigenic system as evident by the development of one precipitin line against corresponding anti-fish-heart antiserum. Both the antigenic fractions contained in fish heart extract were precipitated at 80% and 100% saturation with ammonium sulphate as indicated by the development of 2 precipitin bands against corresponding anti-fish-heart antiserum.

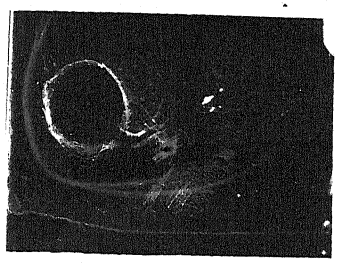
The protein fraction of frog heart extract obtained at 20% saturation with ammonium sulphate did not reveal any precipitin band against corresponding anti-frog-heart antiserum. Protein fraction of frog heart extract obtained at 40% saturation with ammonium sulphate reacted with the development of 2 precipitin bands, similarly the protein fractions of frog heart extract precipitated at 60% and 80% saturation with ammonium sulphate also reacted with the development of 2 precipitin lines, whereas the proteins precipitated by 100% saturation with ammonium sulphate of frog heart extract reacted with the development of 3 precipitin bands against corresponding anti-frog-heart antiserum, untreated frog heart extract also showed 3 precipitin bands; against corresponding anti-frog-heart antiserum. These findings are shown in table 17.

Table - 17 : Effect of salting out with ammonium sulphate on cardiac antigens of frog heart extract.

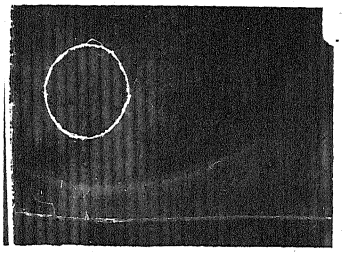
Technique adopted	Number of precipitin bands against various protein fractions of frog heart extract obtained after salting out with different saturations of ammonium sulphate.					
	US Fr.	20%	40%	60%	80%	100%
AGDP	3	0	1	2	2	3
IEP	3	0	1	2	2	3
IPE	3	0	1	2	2	3

US Fr. = Frog heart extract not treated with ammonium sulphate.

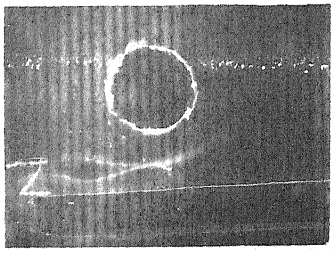
The results of the above experiment thus show that no antigenic system could be isolated after 20% saturation of frog heart extract with ammonium sulphate as indicated by absence of any precipitin line against corresponding anti-frog-heart antiserum. The protein fractions of frog heart extract obtained after 40% saturation with ammonium sulphate had only one antigenic system, however, at 60% and 80% saturation with ammonium sulphate 2 out of 3 precipitin systems could be precipitated, which reacted with the development of two precipitin lines against corresponding anti-frog-heart antiserum. It is apparent that further saturation (100%) of frog heart extract pari passa resulted in the isolation of all the three antigenic components contained



Two precipitin bands obtained after reactivity of anti-tortoise-heart antiserum with tortoise heart antigen (IEP technique).



Single precipitin band obtained after reactivity of anti-tortoise-heart antiserum with tortoise heart antigen exposed to pH 6 for 30 min (IEP technique).



Two precipitin bands obtained after reactivity of anti-tortoise-heart antiserum with tortoise heart proteins obtained at 80% saturation with ammonium sulphate (IEP technique).

in frog heart which reacted with the development of 3 precipitin bands against corresponding anti-frog-heart antiserum in AGDP, IEP and IPE techniques.

Tortoise heart extract proteins obtained after 20% and 40% saturation with ammonium sulphate revealed no precipitin bands on cross reactivity against corresponding anti-tortoise-heart antiserum. The protein fraction of tortoise heart precipitated at 60% and 80% saturation with ammonium sulphate reacted with development of only one precipitin band against anti-tortoise-heart antiserum, whereas the tortoise heart proteins precipitated by 100% saturation with ammonium sulphate reacted with the development of two precipitin bands. These findings are shown in table 18.

Table - 18 : Effect of salting out with ammonium sulphate on cardiac antigens of tortoise heart extract.

Technique adopted	Number of precipitin bands against various protein fractions of tortoise heart extract obtained after salting out with different saturations of ammonium sulphate.					
	UST	20%	40%	60%	80%	100%
AGDP	2	0	0	1	1	2
IEP	2	0	0	1	1	2
IPE	2	0	0	1	1	2

UST = Tortoise heart extract not treated with ammonium sulphate.

These results indicate that the antigenic components contained in tortoise heart extract escape precipitation at 20% and 40% saturation with ammonium sulphate as it is evident by absence of all the precipitin lines in AGDP, IEP and IPE techniques, whereas atleast one of the antigenic components to tortoise heart extract was precipitated after 60% and 80% saturation with ammonium sulphate as indicated by the development of one precipitin band. However, all the antigenic components were found in proteins obtained at 100% saturation with ammonium sulphate as evident by the development of two precipitin bands against anti-tortoise-heart antiserum.

The protein fraction of hen heart extract obtained at 20% saturation with ammonium sulphate, did not show any cross reactivity against corresponding anti-hen-heart antiserum. However, the protein fraction obtained by 40% and 60% saturation of hen heart extract with ammonium sulphate reacted with the development of one precipitin band against corresponding anti-hen-heart antiserum. The protein fraction of hen heart extract at 80% saturation with ammonium sulphate reacted with the development of two precipitin bands and at 100% saturation, the cross reactivity with corresponding anti-hen-heart antiserum showed three precipitin bands. These findings are shown in table 19.

Table - 19 : Effect of salting out with ammonium sulphate on cardiac antigens of hen heart extract.

Technique adopted	Number of precipitin bands against various protein fractions of hen heart extract obtained after salting out with different saturations of ammonium sulphate.					
	USH	20%	40%	60%	80%	100%
AGDP	3	0	1	1	2	3
IEP	3	0	1	1	2	3
IPE	3	0	1	1	2	3

USH = Hen heart extract not treated with ammonium sulphate.

The results of this experiment reveal that all the antigenic components of hen heart escape precipitation at 20% saturation with ammonium sulphate. The 40% and 60% saturation of hen heart extract with ammonium sulphate could isolate only one antigenic component of hen heart which reacted with the development of one precipitin band against corresponding anti_hen-heart antiserum. The protein component of hen heart extract at 80% saturation with ammonium sulphate isolated two out of three antigen systems of hen heart, whereas 100% saturation of hen heart extract resulted in the isolation of all the three antigenic components contained in hen heart as revealed by development of three precipitin bands against corresponding anti-hen-heart antiserum.

The bat heart extract when saturated 20%, 40% and 60% with ammonium sulphate could not isolate any of the antigenic fractions oontained in bat heart as evident by the absence of any precipitin bands, by all three techniques; whereas, the proteins of bat heart extract at 80% saturation with the ammonium sulphate had only one out of three antigenic fractions contained in bat heart, as evident by the development of one precipitin band aginst corresponding anti-bat-heart antiserum. The proteins of bat heart extract 100% saturation with ammonium sulphate showed three precipitin bands against corresponding anti-bat-heart antiserum. These findings are shown in table 20.

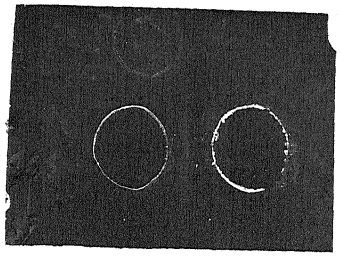
Table - 20 : Effect of salting out with ammonium sulphate on cardiac antigens of bat heart extract.

Technique adopted	Number of precipitin bands against various protein fractions of bat heart extract obtained after salting out with different saturations of ammonium sulphate.					
	USB	20%	40%	60%	80%	100%
AGDP	3	0	0	0	1	3
IEP	3	0	0	0	1	3
IPE	3	0	0	0	1	3

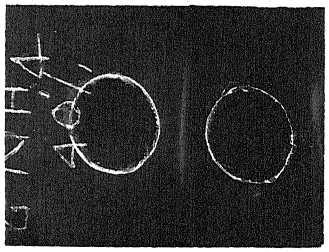
USB = Bat heart extract not treated with ammonium sulphate.

The results of this experiment revealed that all antigenic fractions of bat heart extract escaped precipitation at 20%, 40% and 60% saturation with ammonium sulphate as indicated by the absence of any precipitin line(s) against corresponding anti-bat-heart antiserum. Proteins of bat heart extract obtained at 80% saturation with ammonium sulphate, however, revealed the reactivity with the development of single precipitin line against corresponding anti-bat-heart antiserum, and 100% saturation of bat heart extract with ammonium sulphate resulted in the isolation of all the three antigenic components of bat heart.

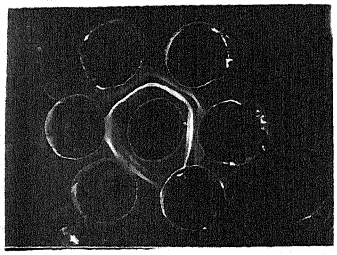
The protein fraction of pig heart extract precipitated by 20% and 40% saturation with ammonium sulphate reacted with the development of one precipitin band, whereas the protein fraction precipitated at 60% saturation reacted with the development of 2 precipitin bands against anti-pig-heart antiserum and the protein component precipitated at 80% and 100% saturation with the ammonium sulphate reacted with the development of only three precipitin bands, whereas the pig heart extract not exposed to ammonium sulphate reacted with the development of 4/5 precipitin bands against corresponding anti-pig-heart antiserum. These findings are shown in table 21.



Single precipitin band obtained after reactivity of anti-pig-heart antiserum with pig heart antigen exposed to 100°C for 30 min (IEP technique); indicating that atleast one of the antigenic fractions of pig heart is thermostable.



Single precipitin band obtained after reactivity of anti-pig-heart antiserum with pig heart proteins precipitated at 40% saturation with ammonium sulphate (IEP technique).



Immune precipitation pattern obtained after cross reactivity of anti-pig-heart antiserum with pig heart antigen differentially salted out by increasing saturation with ammonium sulphate in micro-agar-gel diffusion technique (AGDP).

Table - 21 : Effect of salting out with ammonium sulphate on cardiac antigens of pig heart extract.

Technique adopted	Number of precipitin bands against various protein fractions of pig heart extract obtained after salting out with different saturations of ammonium sulphate.					
	USP	20%	40%	60%	80%	100%
AGDP	4/5	1	1	2	3	3
IEP	4/5	1	1	2	3	3
IPE	4/5	1	1	2	3	3

USB = Pig heart extract not treated with ammonium sulphate.

These results show that 20% and 40% saturation of pig heart extract could isolated one out of 4/5 antigenic components present in pig heart extract, 60% saturation with ammonium sulphate resulted in the isolation of two out of 4/5 antigenic fractions. The proteins of pig heart extract obtained at 80% and 100% saturation with ammonium sulphate had only 3 antigenic fractions, as evident by the development of three precipitin bands against corresponding anti-pig-heart antiserum. It is thus clear from these results that atleast one of the antigenic fraction of pig heart escaped precipitation by ammonium sulphate.

DISCUSSION

DISCUSSION

Comparative anatomy of heart

1. Pisces (Fish) :

The fish heart is considered to be the simplest form among chordates, as the fishes exhibit mainly two chambers, and undivided thin walled atrium or auricle and an undivided ventricle; the sinus venosus and conus arteriousus are also present in fish heart.

2. Amphibian (Frog) :

The heart of amphibians (Frog) is three chambered, two auricles and an undivided ventricle. In addition there is a sinus venosus and a conus arteriosus.

3. Reptiles (Tortoise) :

The reptiles are reported to have four chambered heart, two auricles and ventricle being partially divided into two by a muscular ridge (incomplete septum) extending forward from apex towards the centre. Sinus venosus is also present in reptiles.

4. Avians (Hen) :

Birds have four chambered heart, two auricles and two ventricles. The sinus venosus is absent in birds. The birds maintain high temperature of blood.

5. Mammals (Bat and Pig) :

The heart of mammals has four chambers, two auricles and two ventricles. Sinus venosus is absent.

The comparative anatomy corroborates that during the process of evolution right from pisces and amphibia upto mammalia the chambers of heart increased from two and three to four, rather the heart became more complex.

Analysis of cardiac antigens :

The result of this study reflect that the immunization of rabbits with heterologous heart antigen of different species of animals viz. Fish, Frog, Tortoise, Hen, Bat and Pig resulted in the production of anticardiac antibodies, cross reactive with corresponding cardiac antigen as detected by agar gel diffusion (AGDP), immunoelectrophoresis (IEP) and immune-precipitation electrophoresis (IPE) techniques (Table 2).

Rabbits immunized heterologously with the fish heart antigen responded with the production of anti-fish-heart antibodies (Table 2) demonstrable by the presence of two precipitin bands against fish heart extract in AGDP, IEP and IPE techniques.

Cross reactivity of fish heart antigens against anti-frog-heart antiserum, anti-tortoise-heart antiserum, anti-hen-heart antiserum, anti-bat-heart antiserum and

Table - 22 : Antigenic analysis of cardiac antigen of different animals.

Sl. No.	Name of animals	Species	No. of chambers in heart	No. of antigens contained in heart extract	Probable nature of cardiac antigen
1.	Fish	Pisces	2	2	Protein
2.	Frog	Amphibia	3	3	Protein
3.	Tortoise	Reptilia	4	2	Protein
4.	Hen	Aves	4	3	Protein
5.	Bat	Mammalia	4	3	Protein
6.	Pig	Mammalia	4	4/5	Protein + 1 to 2 other than protein (polysaccharide ??)

anti-pig-heart antiserum in AGDP, IEP and IPE revealed that anti heart antiserum of any of these species did not cross react with fish heart antigen (Table 3). Similarly the anti-fish-heart antiserum did not show any cross reactivity with antigens of frog, tortoise, hen, bat and pig heart indicating thereby that the fish heart antigens are species specific and not shared by the cardiac antigens of other animal species included in this study.

Immunization of rabbits with frog heart antigen evoked the development of circulating anti cardiac antibodies reactive with corresponding cardiac antigen to produce three precipitin bands in AGDP, IPE and IEP techniques (Table 2).

The frog heart antigen failed to show any cross reactivity against anti heart antibodies of various species viz. Fish, Tortoise, Hen, Bat and Pig. Similarly the anti-frog-heart antiserum did not show any cross reactivity with cardiac antigens of fish, tortoise, hen, bat and pig (Table 3). It would suggest that the frog heart antigens are also species specific and are not shared with the cardiac antigens of any other animal under study.

The administration of multiple doses of tortoise heart antigen into rabbits resulted in the development of circulating anti heart antibodies which showed two precipitin bands against tortoise heart extract in AGDP, IEP and IPE techniques (Table 2).

The tortoise heart antigens did not cross react against the anticardiac antibodies of any other animal species viz. fish, frog, hen, bat and pig. Similarly the anti-tortoise-heart antiserum did not show any cross reactivity with cardiac antigens of other animal species (Table 3), indicating thereby that the tortoise heart antigens are not shared by other animals, and are therefore species specific.

The immunization of rabbits with hen heart antigen resulted in the production of anti-hen-heart antibodies reacting with the development of 3 precipitin bands against hen heart extract in AGDP, IEP and IPE techniques (Table 2).

Cross reactivity of hen heart antigens with anti-cardiac antibodies of different animals viz. Fish, Frog, Tortoise, Bat and Pig did not show any reaction and the hen heart antigen only cross reacted with corresponding anti-hen-heart antibodies (Table 3).

The immunization of rabbits with bat heart antigen evoked the development of anti-bat-heart antibodies which cross reacted with corresponding cardiac antigen to give three precipitin bands in AGDP, IEP and IPE techniques (Table 2). Bat heart antigens were not cross-reactive against anti heart antibodies of any other species, than anti-bat-heart antiserum. Similarly the anti-bat-heart antibodies could not react with any other antigen viz.

Fish, Frog, Tortoise, Hen and Pig heart antigens. These antibodies were readtive only against correspond ng bat heart antigen (Table 3).

The rabbits immunized with pig heart antigen responded with the development of multiple anti-pig-heart antibodies directed against rabbit cardiac antigens as detected by development of four to five precipitin bands in AGDP, IEP and IPE techniques (Table 2).

These antigenic components of pig heart when subjected to cross reactivity with anti heart antibodies of different animals viz. Fish, Frog, Tortoise, Hen and Bat did not reveal any reaction against anti heart antibodies of any other species of animals. In the same way the anti-pig-heart antibodies did not cross react with the heart antigens of any other animal species; even no cross reactivity was observed with bat-heart antigens which is also a mammal (Table 3). These results indicate that pig heart antigens are species specific and are not shared with the cardiac antigens of any other animal under study.

Attempts at the production of anticardiac antibodies in experimental animals have met with varying sucess. Kaplan (1958a and 1958b) demonstrated the development of anticardiac antibodies after immunization of rabbits with homogenates of beaf heart. Gery et al. (1960) have also

demonstrated the development of heart specific antibodies against homologous and heterologous heart antigens. Kaplan and Craig (1961, 1963), Ehrenfeld (1961), Davies (1964), Chaturvedi and Mehrotra (1967), Halbert (1970), Chaturvedi and Gupta (1971), Gupta(1976 and 1977) have also reported that immunization of rabbits with homologous or heterologous cardiac antigens with or without Freund's adjuvant resulted in the production of circulating anticardiac antibodies. All these studies are confined to the use of only mammalian heart antigens for the immunization of rabbits. These results are similar to those obtained in the present study that the rabbit immunized with pig heart antigen developed circulating anticardiac antibodies cross reactive with pig heart extract. In the present study, anticardiac antibodies have also been produced against cardiac antigens of other chordates viz. fish, frog, tortoise, hen and bat cross reactive with corresponding cardiac antigen. So far there has been no report in literature on the cardiac antigens of chordates other than higher mammals; therefore it is difficult to compare the results of this study.

Further characterization of heart antigens was done by exposing the heart extract to heat, pH and differential saturation with ammonium sulphate. The findings of heat susceptibility of cardiac antigens of different species of animals have been shown in detail in table 23.

Table - 23 : Effect of heat on different cardiac antigens.

Animal species	Number of precipitin bands at various Temperature (°C)					
	UET	55°C	60°C	70°C	80°C	100°C
Fish	2	1	1	0	0	0
Frog	3	1	1	1	0	0
Tortoise	2	0	0	0	0	0
Hen	3	2	1	1	1	1*
Bat	3	2	1	0	0	0
Pig	4/5	4	2	2	1	1

*Shows faint precipitin line.

UET = Cardiac heart antigen not exposed to heat.

It was observed that out of two antigenic components contained in fish heart one was highly heat labile and was destroyed even at 55°C within 30 minutes whereas the other component was stable upto 60°C (Fig 4). Two out of three antigenic components of frog heart were found susceptible to a temperature of 55°C for 30 min whereas one of components resisted the temperature upto 70°C, beyond which all components were destroyed (Fig 5). Both the antigenic systems contained in tortoise heart were found extremely heat labile as they were destroyed even at 55°C within 30 minutes (Fig 6).

One of the antigenic fractions of hen heart was destroyed after exposure to 55°C within 30 minutes, whereas the other two could withstand this temperature, the third antigenic component was destroyed more slowly and even after exposure to 100°C faint reactivity persisted (Fig 7).

The bat heart antigens revealed that out of three, one was destroyed after exposure to 55°C for 30 minutes whereas the other two could withstand this temperature. Only one antigenic component could withstand temperature upto 60°C for 30 minutes and all were destroyed after exposure to 80°C or more within 30 minutes (Fig 8).

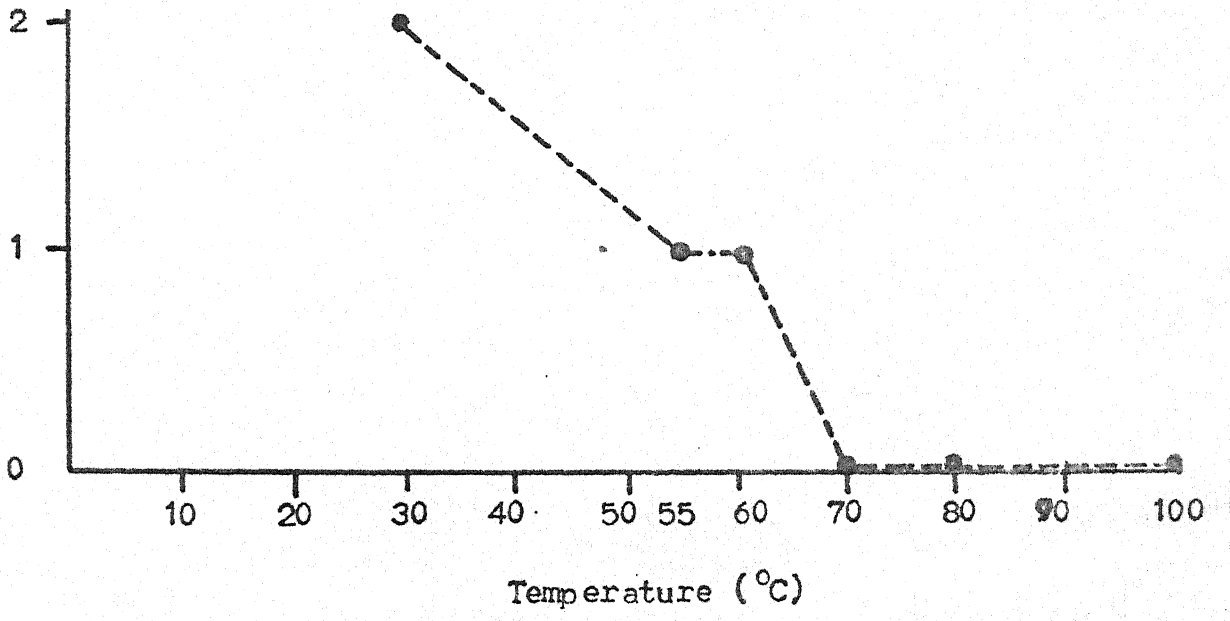


Fig 4 : Effect of temperature on cardiac antigens of fish

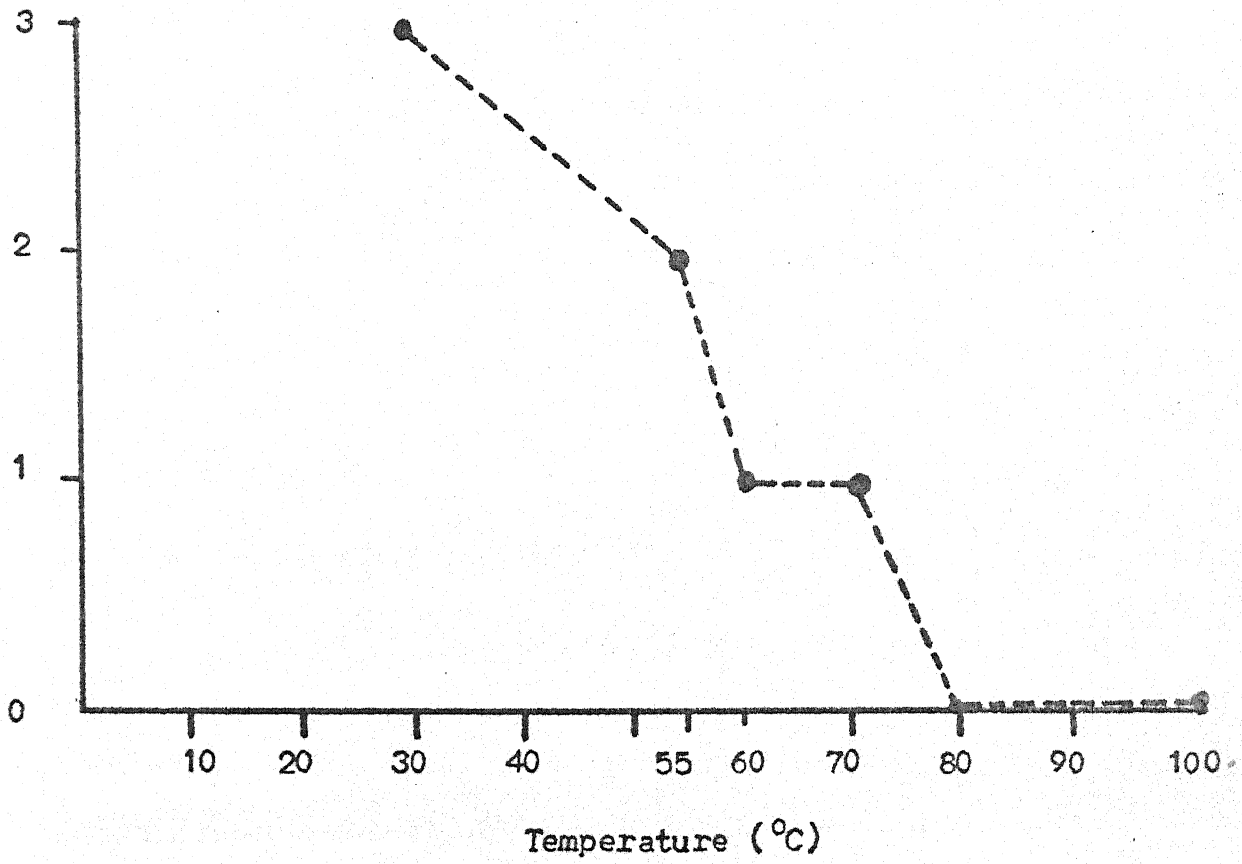


Fig 5 : Effect of temperature on cardiac antigens of frog.

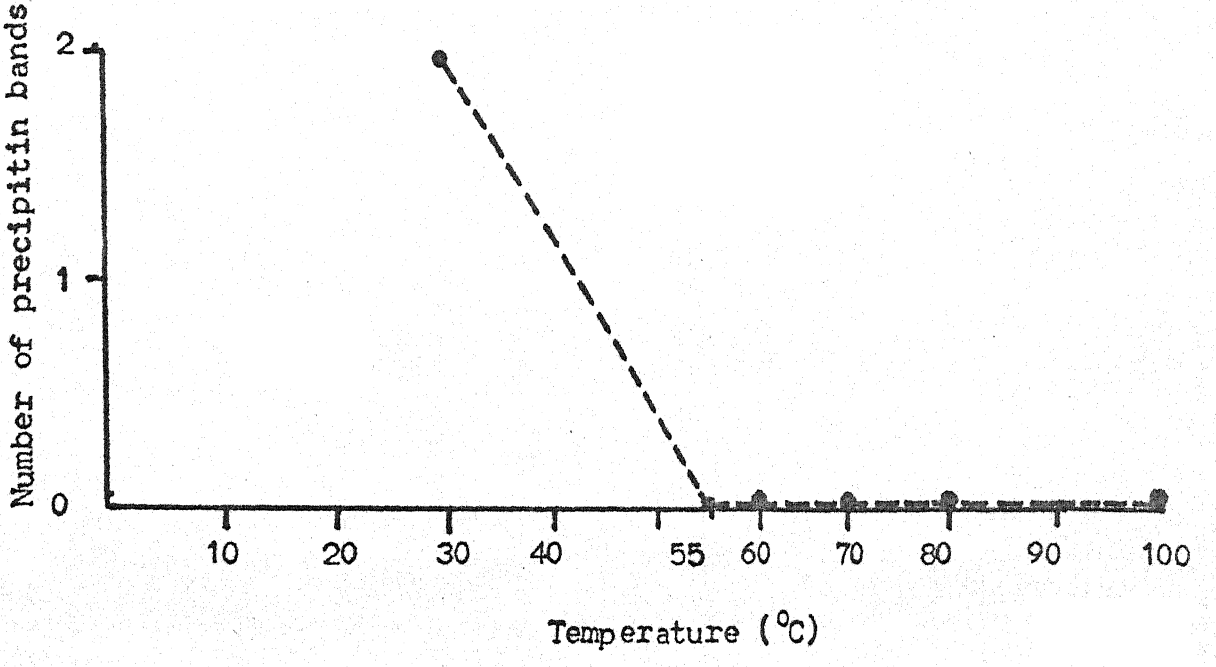


Fig 6 : Effect of temperature on cardiac antigens of tortoise.

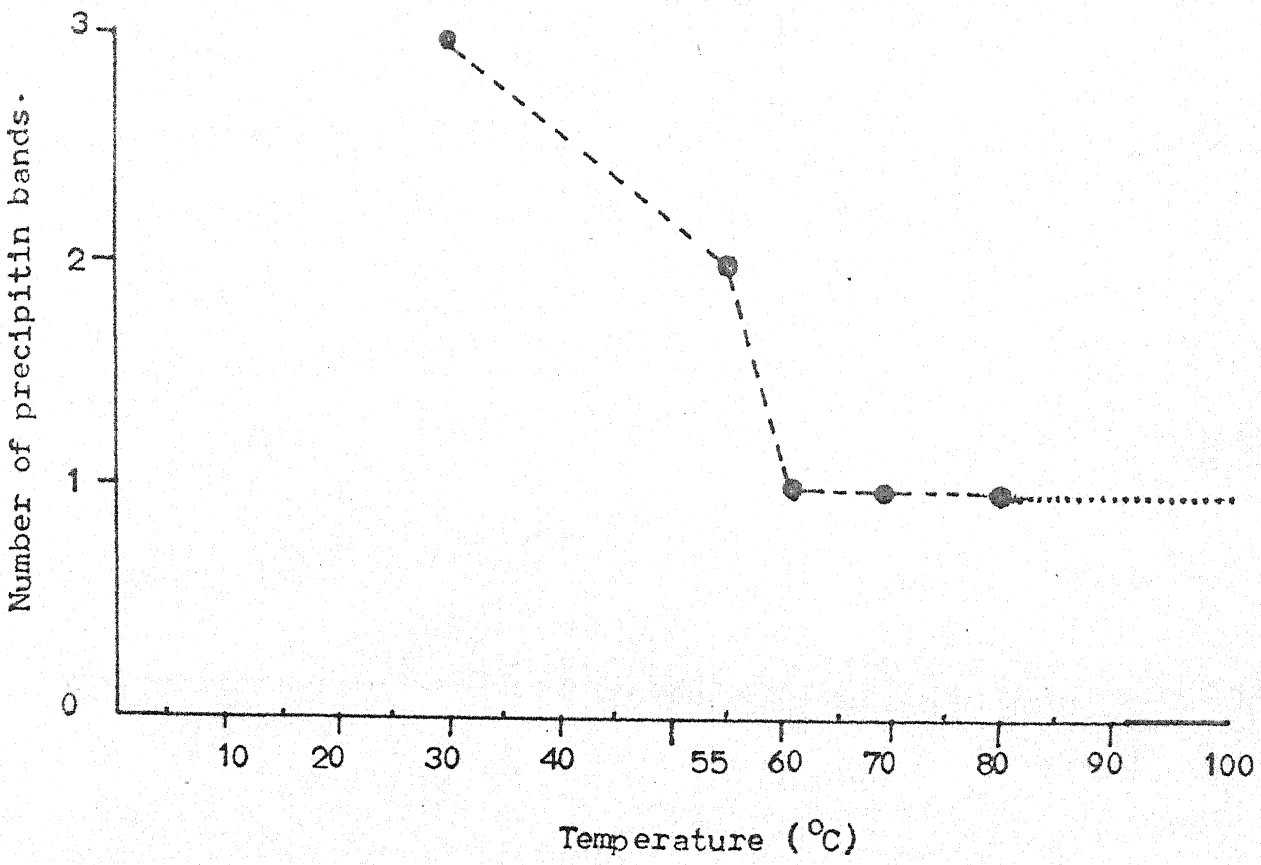


Fig 7 : Effect of temperature on cardiac antigens of hen.

..... = Shows faint precipitin line

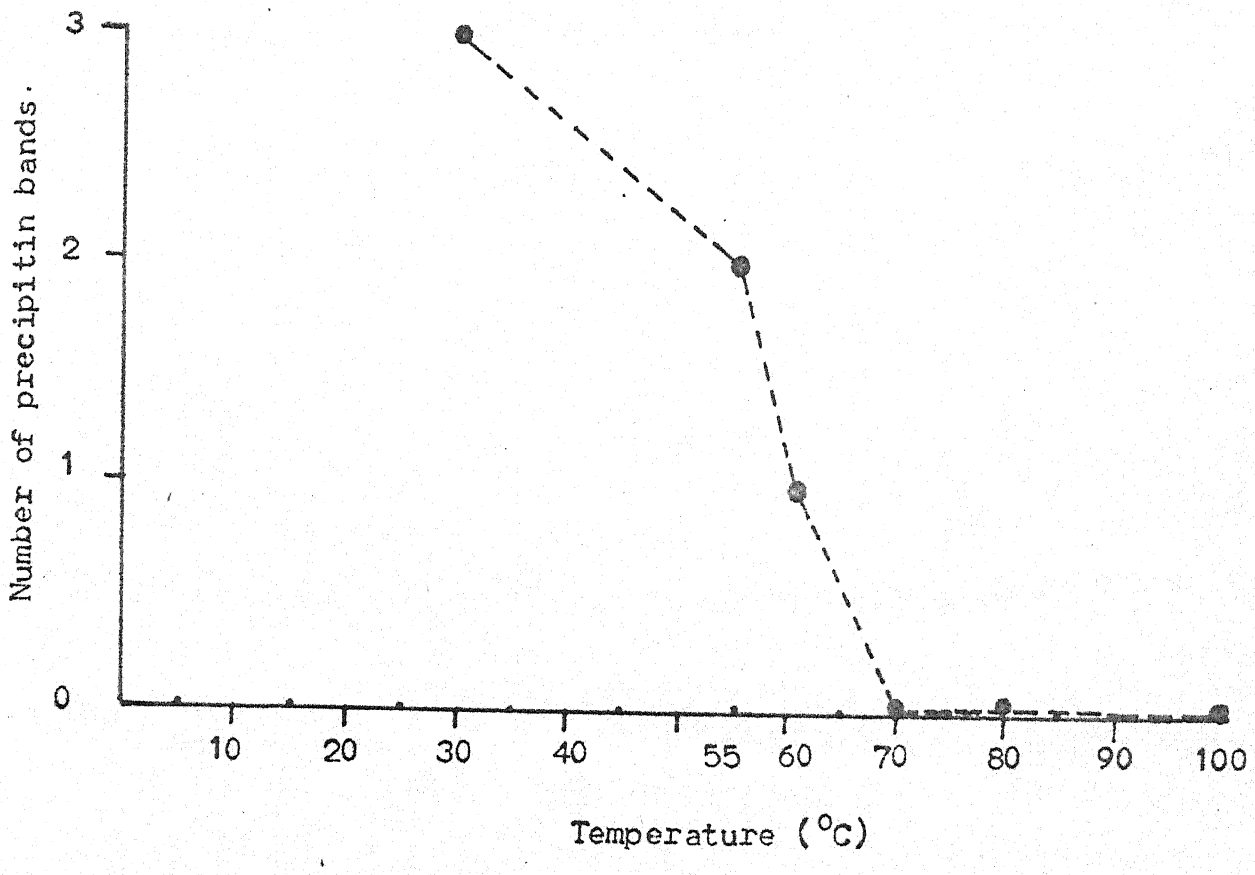


Fig 8 : Effect of temperature on cardiac antigens of bat.

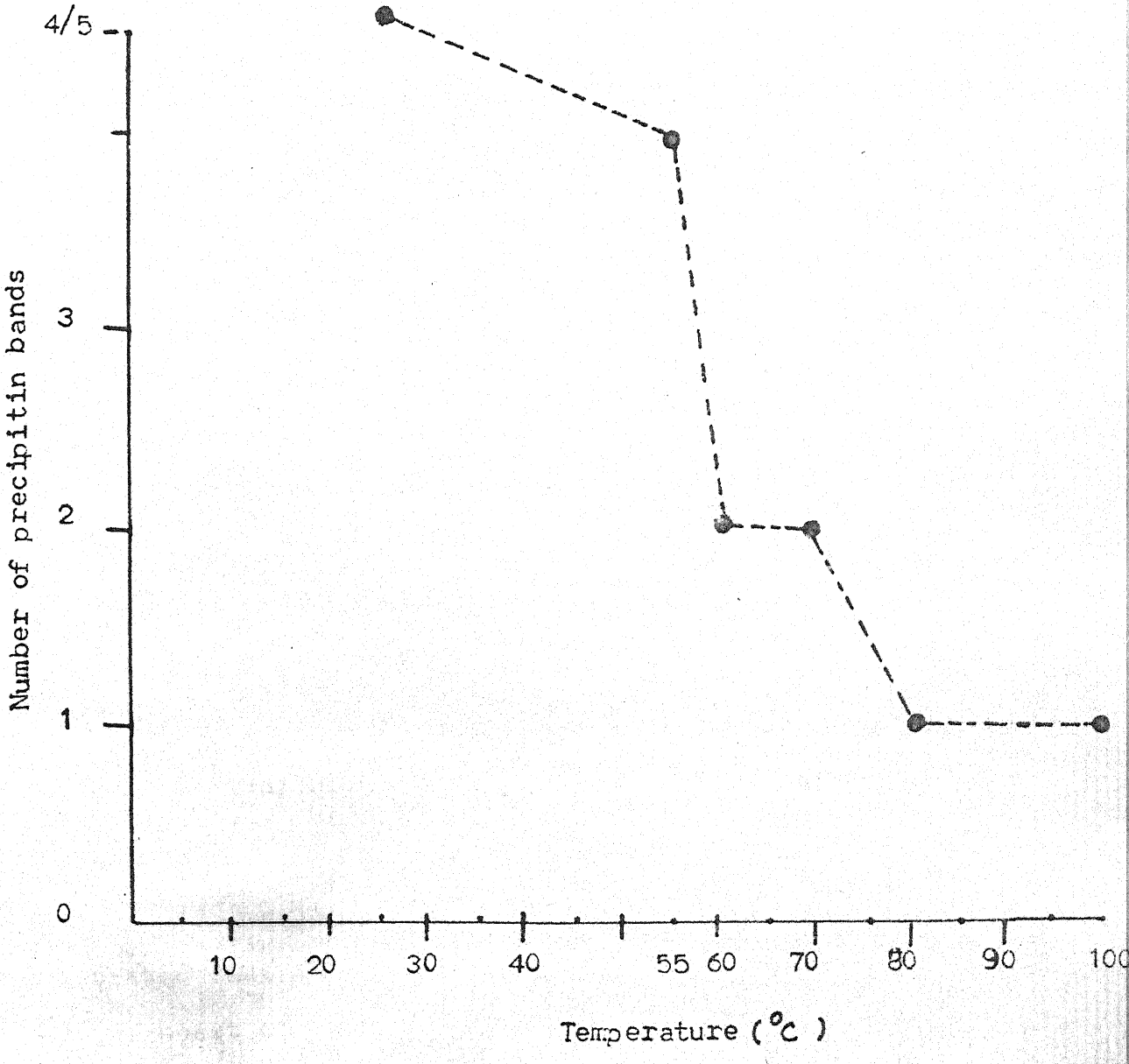


Fig) : Effect of temperature on cardiac antigens of pig.

Thermal susceptibility of the pig heart antigens revealed that one was destroyed after exposure to 55°C within 30 minites, two resisted the temperature of 60°C and 70°C upto 30 minutes, however, atleast one fraction could withstand higher temperature and as a result, reactivity persisted even after exposure to 100°C (Fig 9).

Holm and Halbert (1970) have reported that the antigens contained in the heart of most of the mammals are heat susceptible and are inactivated at temperature of 85°C or more.

Holm et al (1972), while working with human heart antigens, have reported that some of the fractions were fairly thermoresistant and faint reactivity persisted even after exposure to 100°C temperature. Thus the thermal behaviour of pig heart antigens, as observed in the present study, has close similarity to that of the thermal behaviour of human heart antigens (Holm et al 1972).

Natu et al (1980) have also demonstrated that all the antigens contained in rat heart were destroyed at a temperature of 90°C or more.

The results of the present study have also demonstrated that majority of antigenic components contained in the heart of various animals under study were thermolabile

except for a minor component of hen heart and also of pig heart which retained faint reactivity even after exposure to 100°C for 30 minutes.

The persistance of reactivity of atleast one of the pig heart antigen even after exposure to 100°C for 30 minutes suggested that this component may not be protein in nature.

Persistance of faint reactivity of atleast one of cardiac antigens contained in hen heart might be explained due to atmospheric adoptation of birds, as they maintain high temperature of blood as compared to other animals.

There are no reports in the literature on the thermal behaviour of cardiac antigens of lower chordates, hence it is difficult to compare the findings of present study.

The findings of the pH susceptibility of cardiac antigens of different species of animals have been summarized in table 24.

The fish heart antigens were relatively stable at neutral and alkaline pH (9.5) as compared to acidic pH (4.5), when one of the antigens was lost (Fig 10); indicating therel that atleast one of the antigen contained in fish heart is destroyed at moderately acidic pH.

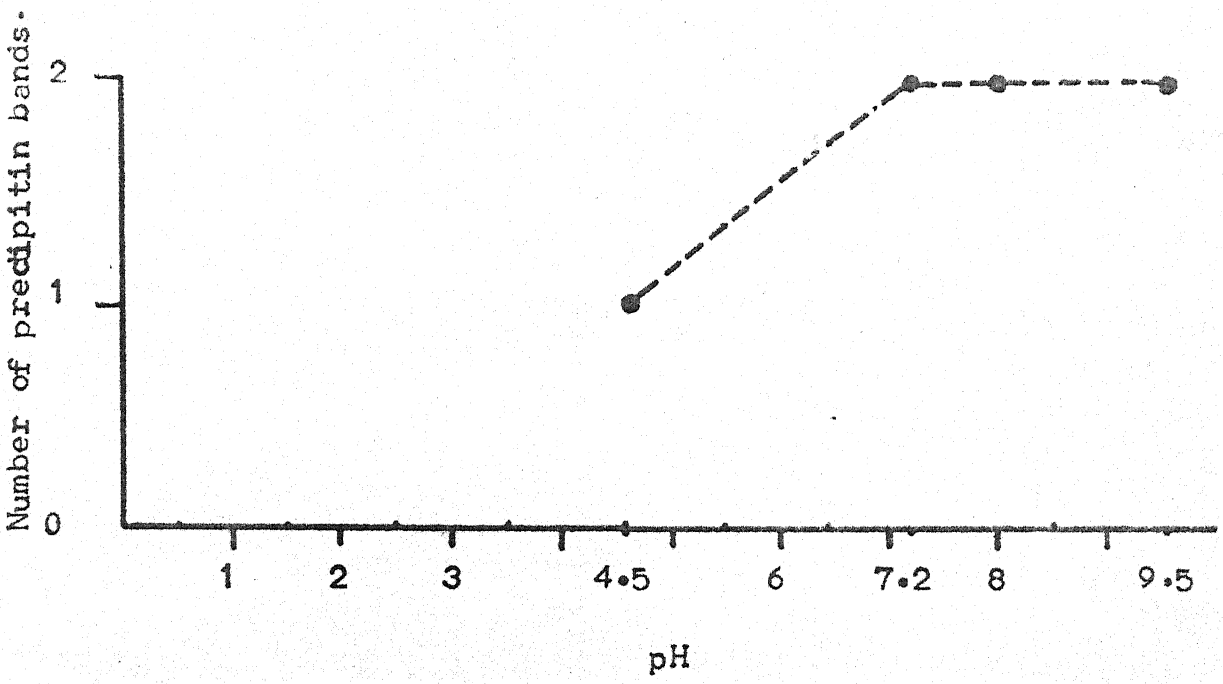


Fig 10 : Effect of pH on cardiac antigens of fish.

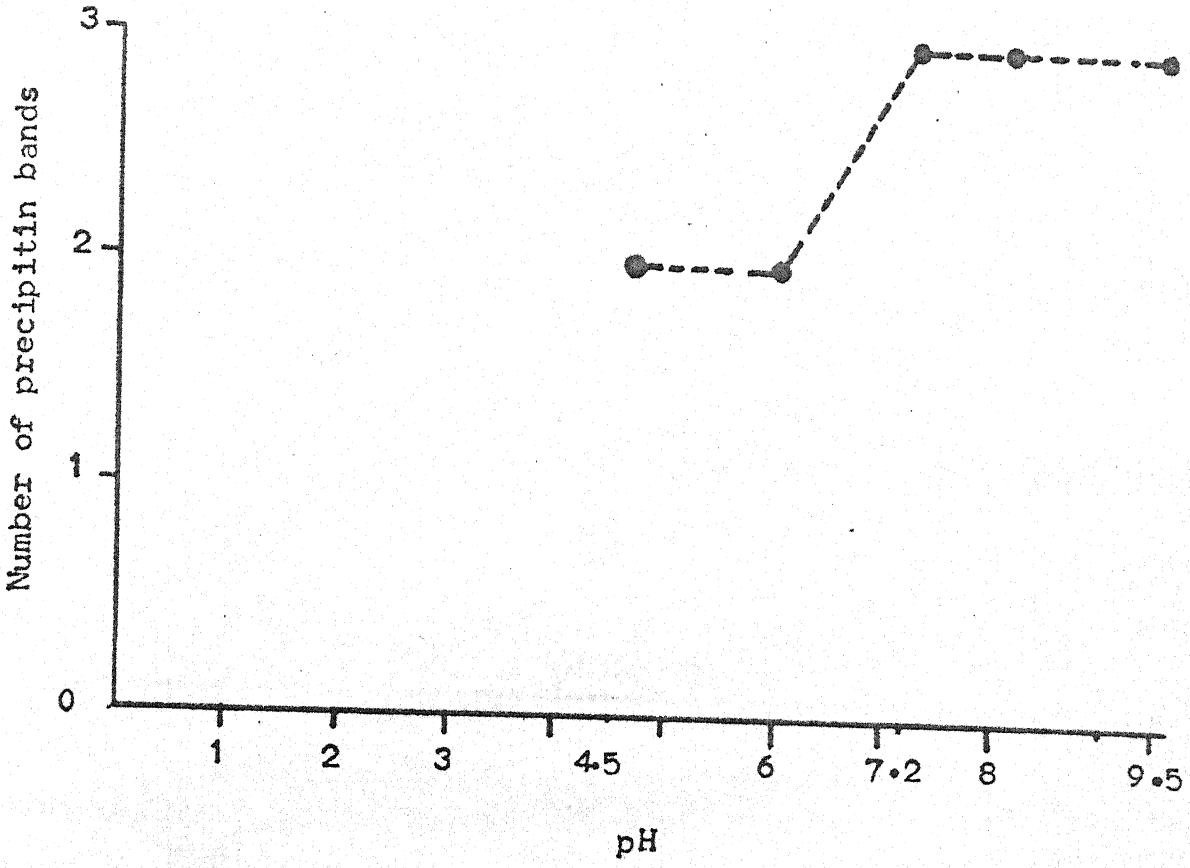


Fig 11 : Effect of pH on cardiac antigens of frog.

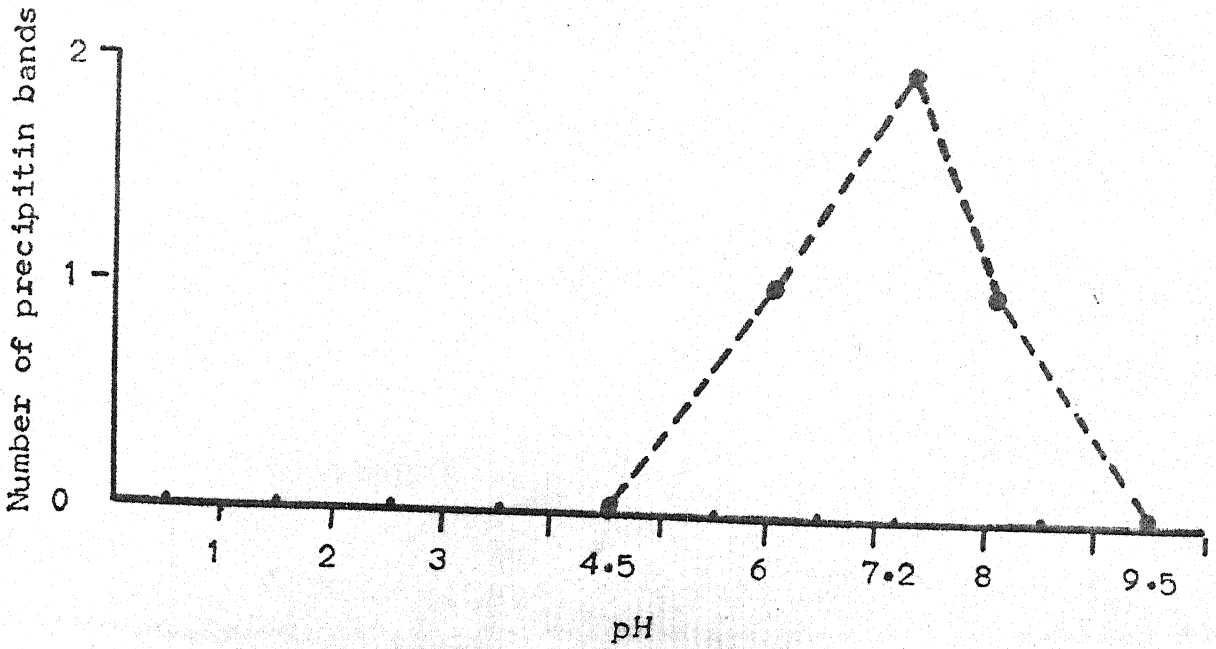


Fig 12: Effect of pH on cardiac antigens of tortoise.

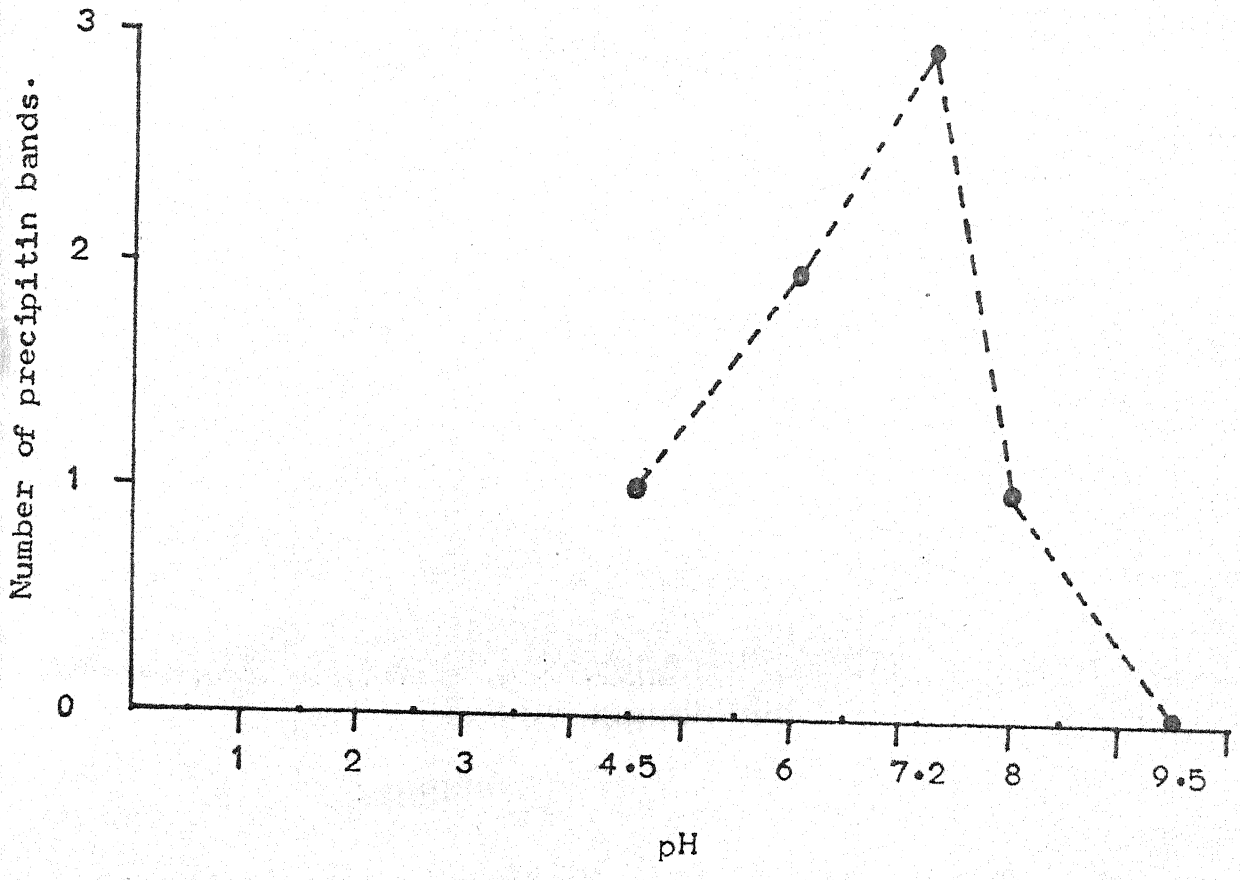


Fig 13 : Effect of pH on cardiac antigens of hen.

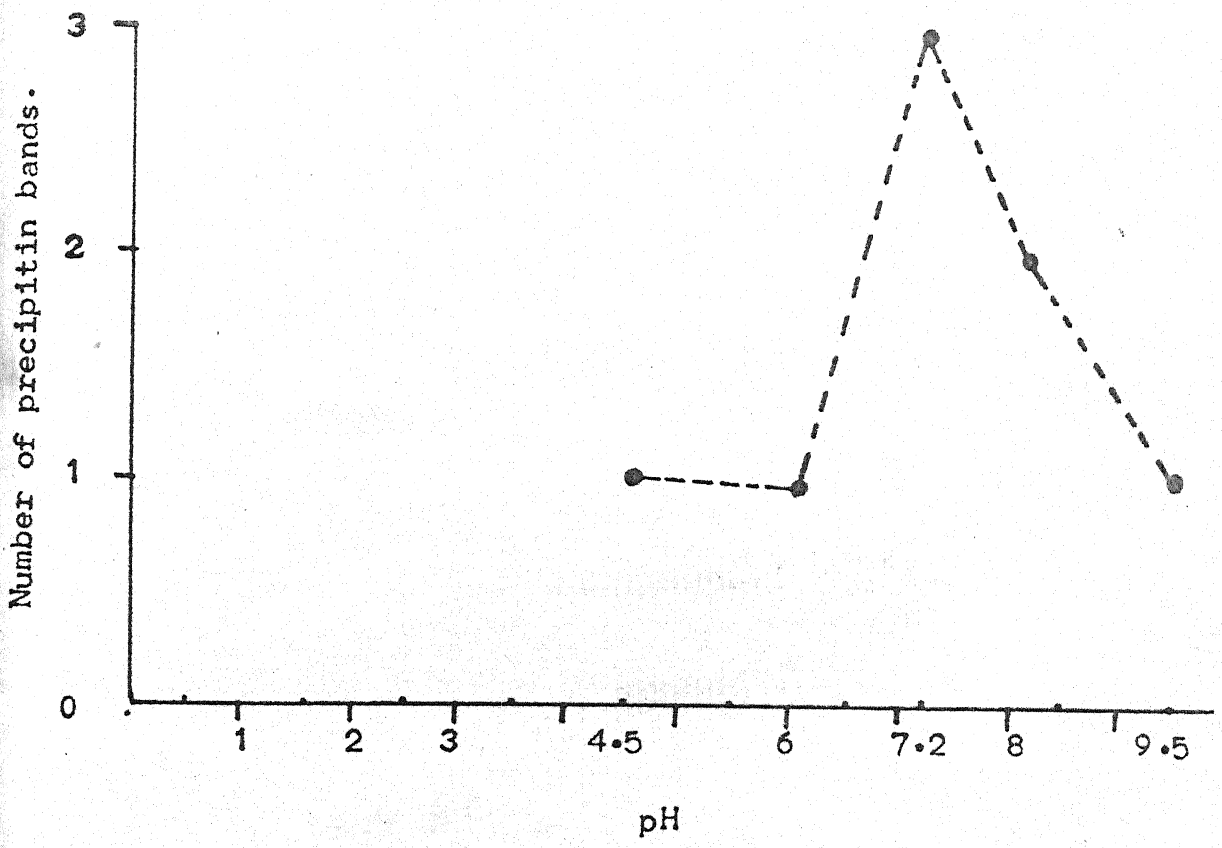


Fig 14 : Effect of pH on cardiac antigens of bat.

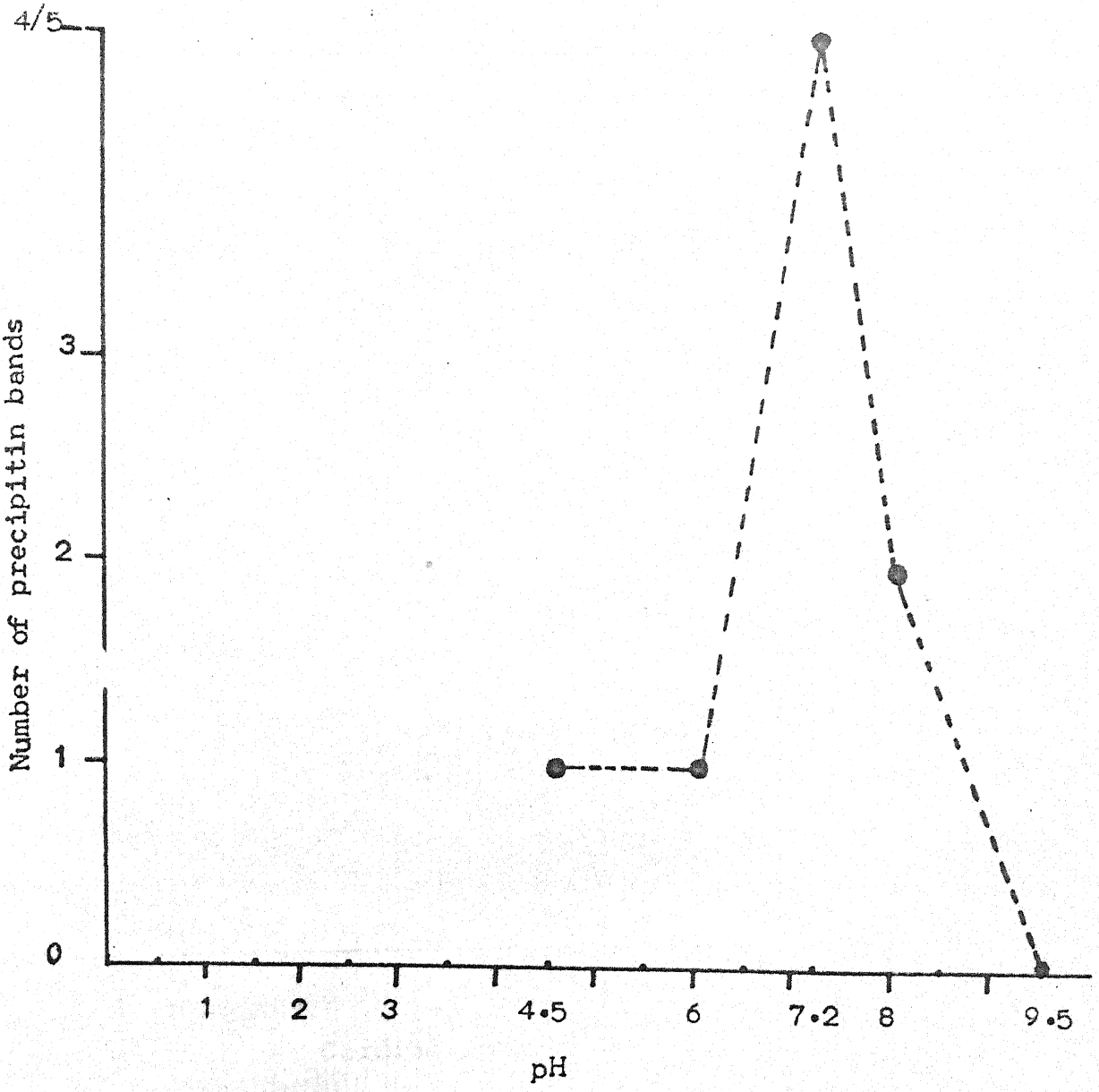


Fig 15 : Effect of pH on cardiac antigens of pig.

Table - 24 : Effect of pH on different cardiac antigens.

Animal species	Number of precipitin bands at various pH				
	UEP	4.5	6	8	9.5
Fish	2	1	2	2	2
Frog	3	2	2	3	3
Tortoise	2	0	1	1	0
Hen	3	1	2	1	0
Bat	3	1	1	2	0
Pig	4/5	1	1	2	0

UEP = Cardiac antigen at pH 7.2.

All the frog heart antigens were found optimally active at neutral or moderately alkaline pH viz 7.2, 8 and 9.5, whereas at moderately acific pH (4.5) one of the antigens was lost (Fig 11).

The antigenic systems present in tortoise heart were highly susceptible to both moderately acidic as well as moderately alkaline pH as both the fraction were destroyes at pH 4.5 and 9.5 respectively. At moderately acidic or alkaline pH (6 or 8) one of the antigens was lost. Both the antigenic components of tortoise heart were optimally active around neutral or slightly alkaline pH viz 7.2 and 8 respectively (Fig 12).

Hen heart antigen was found to have varying susceptivity to pH, viz. at pH 4.5 and pH 8 one of the cardiac antigens was lost; the antigens were found optimally active at pH 7.2, whereas at pH 9.5 no reactivity was observed (Fig 13). It is apparent that the activity of hen heart antigen decreased at alkaline and acidic pH.

The bat heart antigen was found to be optimally reactive at pH 7.2 and 8 and less reactive at acidic pH 4.5 and alkaline pH 9.5 (Fig 14).

The pig heart antigens were maximally reactive at pH 7.2 and 8 ; comparatively less reactivity was observed at pH 4.5 and 6, whereas all reactivity was lost at highly alkaline pH (9.5) (Fig 15).

Holm and Halbert et al (1970) have reported that antigenicity of rabbit heart is destroyed at extremely low pH (1) and extremely high pH (12 or more). Lin et al (1972a and 1972b) while working with human heart antigens have reported the loss of antigenic reactivity at or below pH 5, at at pH 10 or more.

Natu et al (1980) have reported that all the antigens contained in rat heart were destroyed at extremes of pH and majority of antigens were lost beyond a pH of 9.0, however, surprisingly enough, one of the antigens of rat heart, in their study retained its activity at pH as high as 1. These authors have not advanced any comments to explain such a strange behaviour of one of the antigens contained in rat heart. Although there are no reports in the literature on the pH susceptibility of cardiac antigens of lower chordates, the general behaviour of these antigens, as far as pH susceptibility is concerned, appears to be closely similar to those of mammalian heart antigens as observed in the present as well as proceeding studies (Holm and Halbert 1970, Lin et al 1972a, 1972b and Natu et al 1980).

Table - 25 : Effect of salting out with ammonium sulphate on different cardiac antigens.

Animal Species	Number of precipitin bands at various saturations with ammonium sulphate.					
	UES	20%	40%	60%	80%	100%
Fish	2	0	1	1	2	2
Frog	3	0	1	2	2	3
Tortoise	2	0	0	1	1	2
Hen	3	0	1	1	2	3
Bat	3	0	0	0	1	3
Pig	4/5	1	1	2	3	3

UES = Cardiac antigen not treated with ammonium sulphate.

Out of two antigenic systems contained in fish heart, one was isolated at 40% and 60% saturations with ammonium sulphate, whereas the protein precipitated at 100% saturation contained both antigenic fractions (Fig 16). It would thus suggest that fish heart antigens basically are protein in nature.

Frog heart antigens were salted out variously with ammonium sulphate; at 20% saturation no antigenic fraction could be obtained, at 40%, one of the antigens was isolated; at 60% and 80% two of the three antigens were precipitated, whereas 100% saturation with ammonium sulphate isolated all the antigenic components of frog heart (Fig 17). It would suggest that all the antigenic components present in frog heart were protein in nature.

Comparatively lower saturation with ammonium sulphate could not isolate any of the tortoise heart antigens; at 60% and 80% saturations, one antigenic component was isolated, whereas both the antigenic fractions were isolated at 100% saturation with ammonium sulphate (Fig 18). These observations reveal that all the antigenic fractions contained in tortoise heart were protein in nature.

The antigenic fractions, contained in hen heart were not precipitated at 20% saturation with ammonium sulphate. It was partially precipitated at 40% and 60%

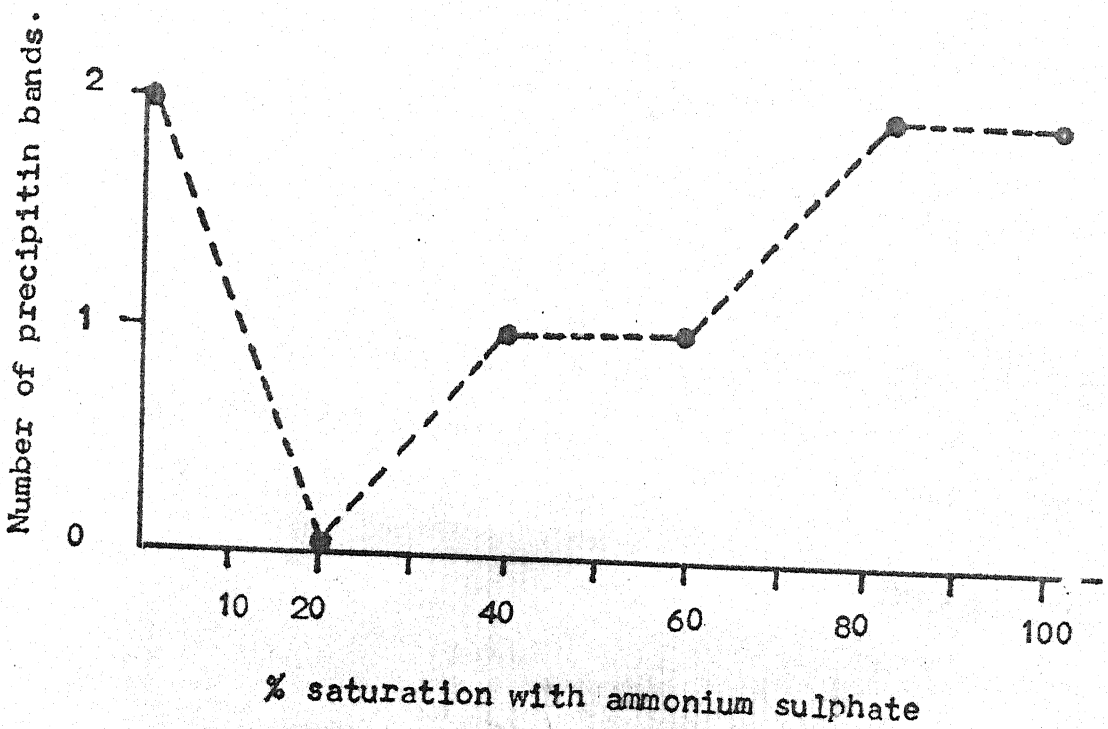


Fig 16 : Effect of salting out with ammonium sulphate on cardiac antigens of Fish.

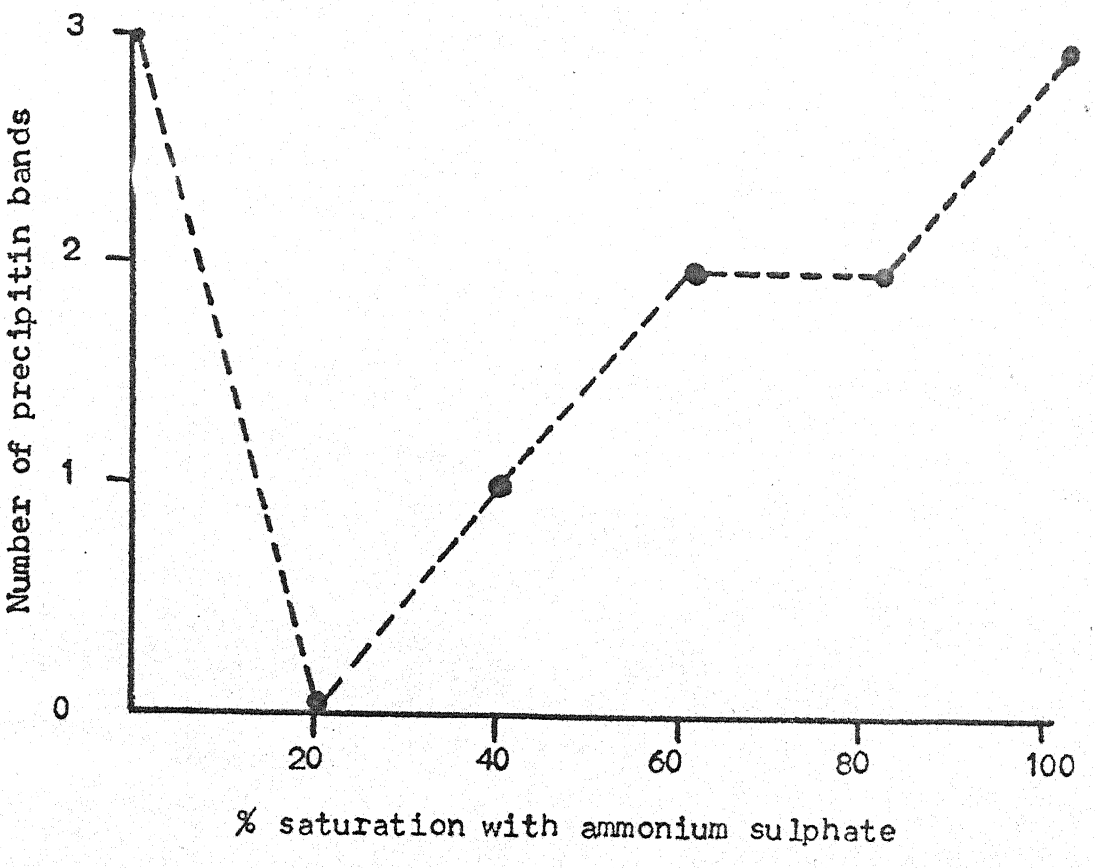


Fig 17: Effect of salting out with ammonium sulphate on cardiac antigens of frog.

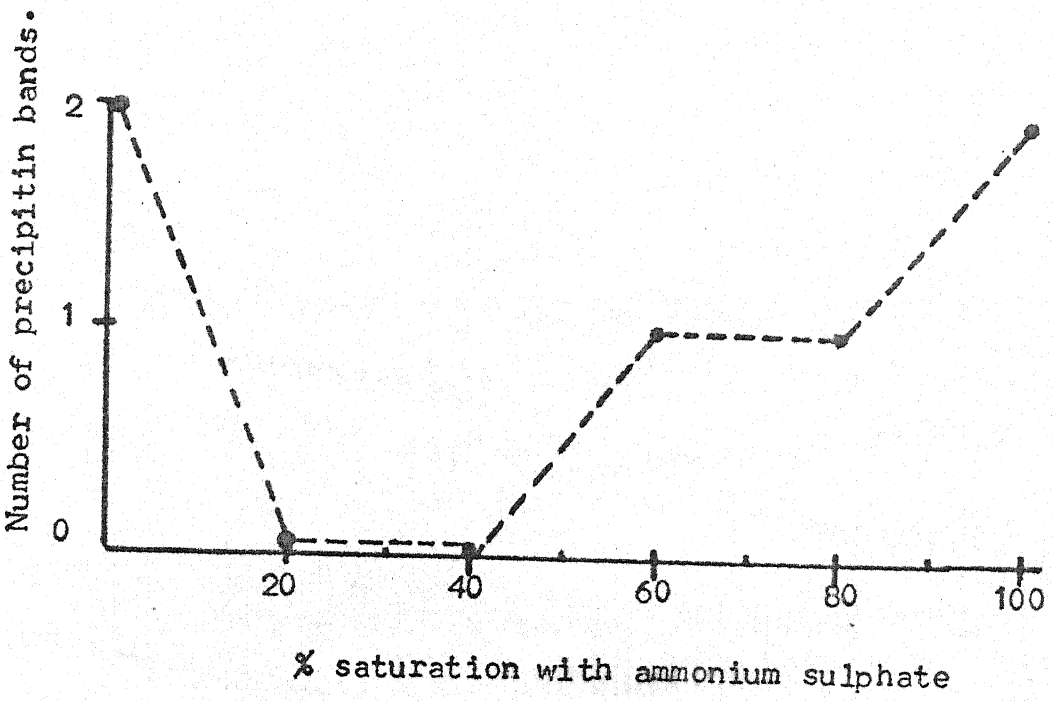


Fig 18: Effect of salting out with ammonium sulphate on cardiac antigens of tortoise.

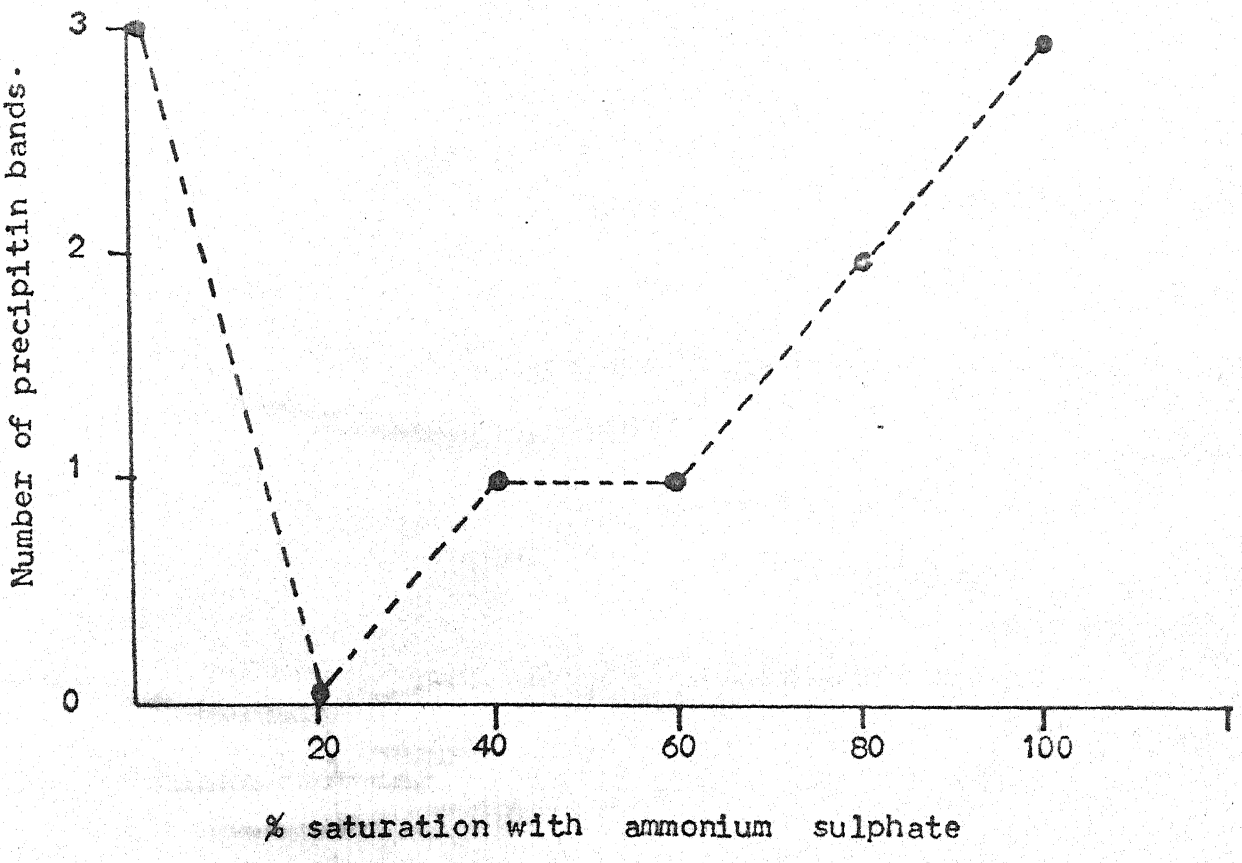


Fig 19 : Effect of salting out with ammonium sulphate on cardiac antigens of hen.

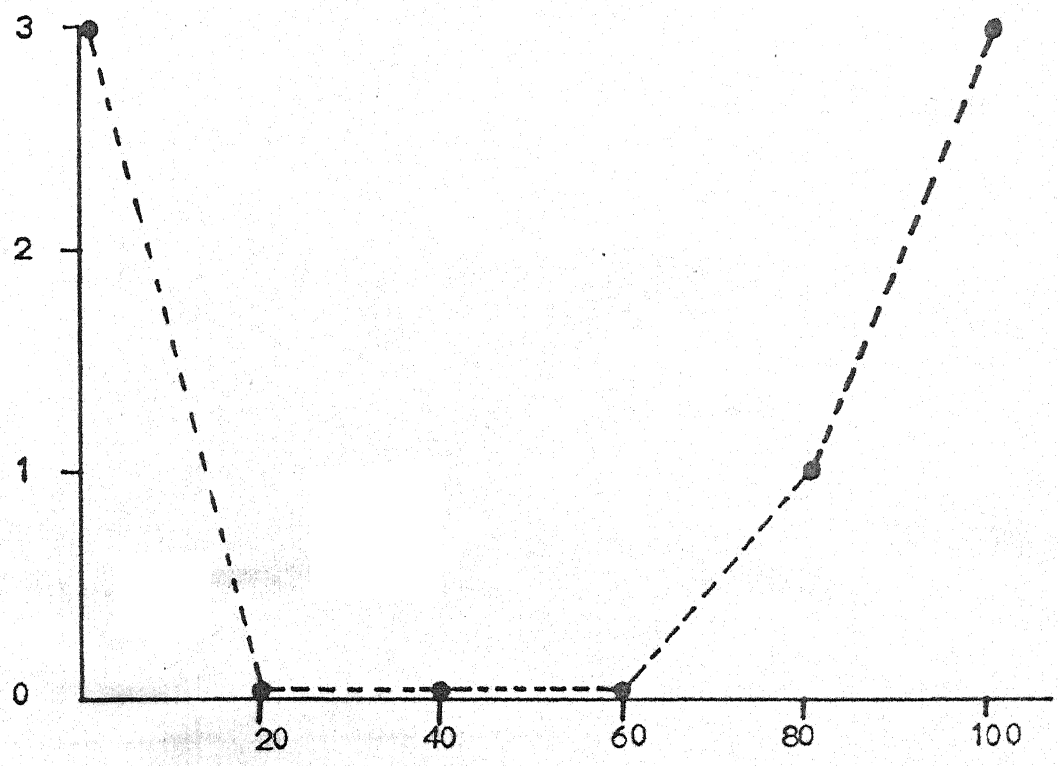


Fig 20: Effect of salting out with ammonium sulphate on cardiac antigens of bat.

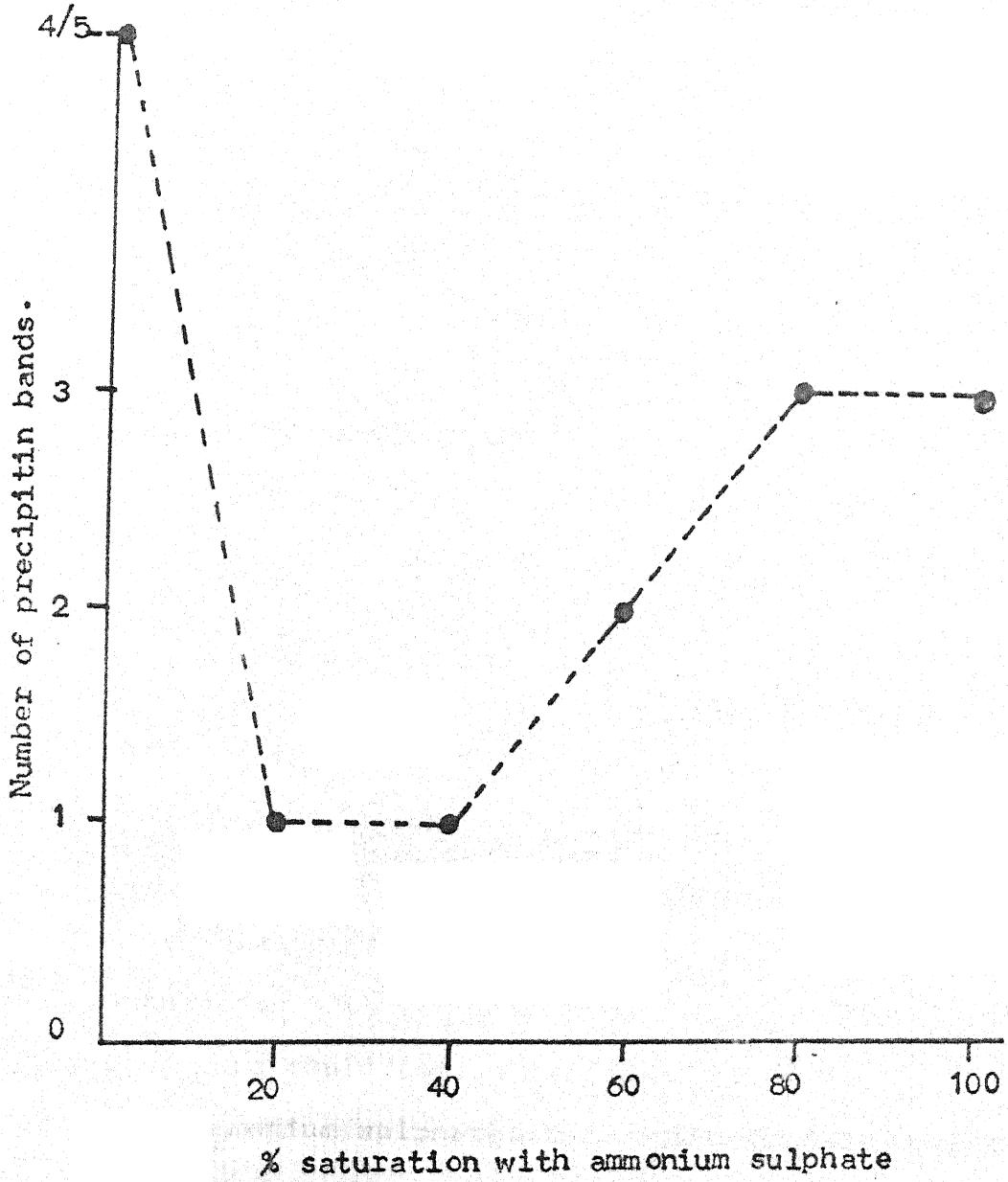


Fig 21 : Effect of salting out with ammonium sulphate on cardiac antigens of pig.

saturation with ammonium sulphate whereas all the fractions were completely isolated at 100% saturation with ammonium sulphate (Fig 19); which indicated that all the antigenic systems present in hen heart were protein in nature.

The bat heart antigen was salted out variously with ammonium sulphate. By precipitation at 20%, 40% and 60% saturation with ammonium sulphate no antigenic fractions could be isolated, whereas increasing saturation with ammonium sulphate viz 80%, resulted in isolation of one antigenic fraction, moreover all the components were isolated at 100% saturation with ammonium sulphate (Fig 20). It indicates that all the antigenic fractions contained in bat heart are protein in nature.

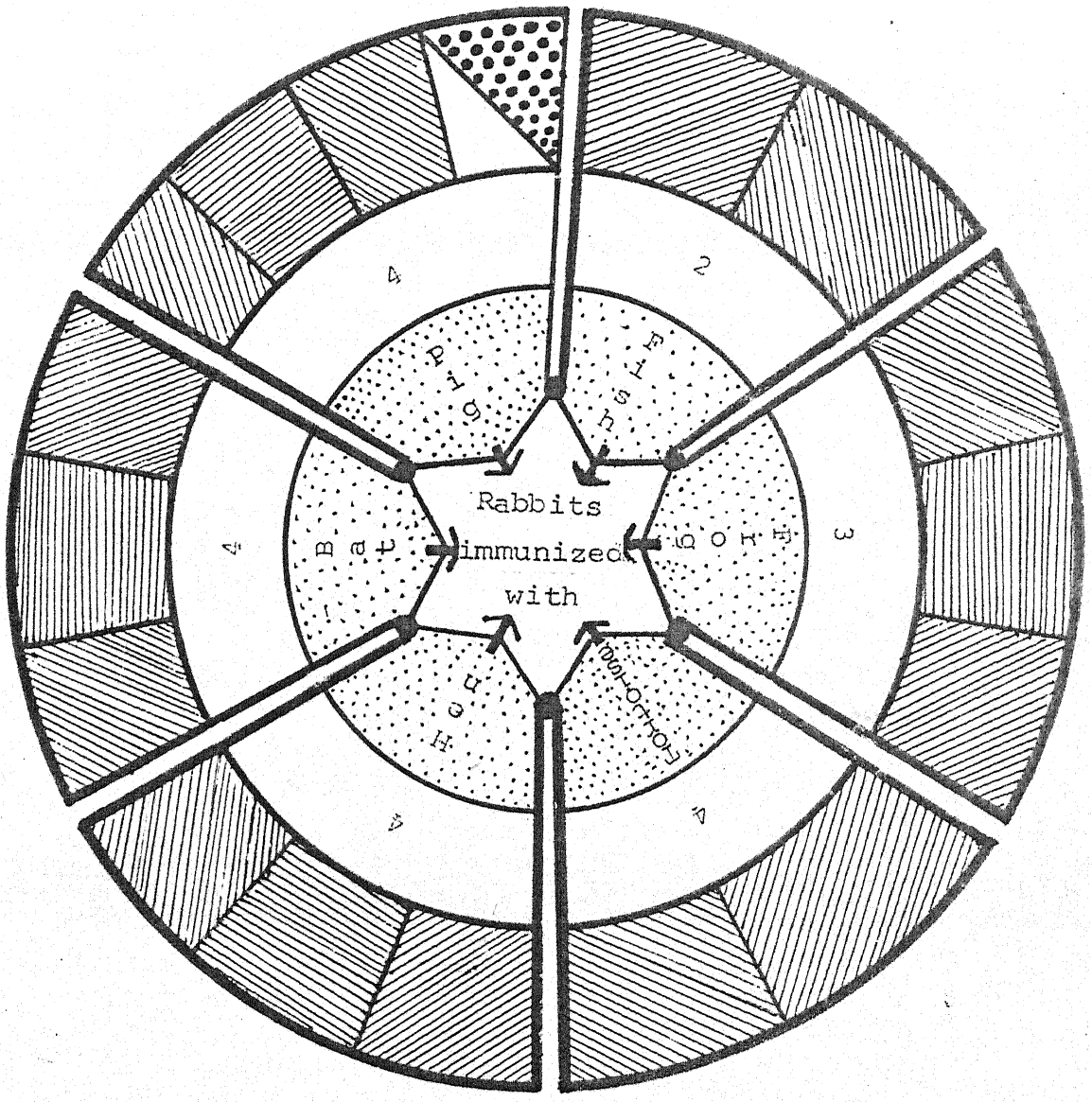
Salting out of pig heart antigens with ammonium sulphate revealed that only one of the fraction was salted out at 20% and 40% saturations with ammonium sulphate, at 60% saturation two fraction were isolated, whereas only three antigenic fractions could be isolated even at 100°C saturation with ammonium sulphate; thus one to two antigenic component could not be precipitated even at 100°C saturation ith ammonium sulphate, indicating thereby that these fractions may not be protein in nature.

Thus all the antigens contained in the heart of lower chordated appear to be protein in nature. There are no

similar studies on the salting out behavious of cardiac antigens of lower vertebrates in the literature, hence it is difficult to compare these results.

Holm et al (1970) working with rabbit heart antigens have also demonstrated that out of five antigenic systems contained in rabbit heart, atleast three were protein in nature and could be salted out with ammonium sulphate and potassium phosphate. Chaturvedi et al (1971) while working with monkey heart have also reported that these antigens are mostly protein in nature. Lin et al (1972a, 1972b) have also demonstrated that majority of human heart antigens are protein in nature. Chaturvedi et al (1973), Gupta (1976) and Natu and Chaturvedi (1977) working with rat heart have also reported that the rat heart antigens were mostly protein in nature.

The findings of the present study on pig heart antigens have clearly revealed that besides protein antigens there is atleast one antigenic component which is thermostable. Holm et al (1970), Chaturvedi et al (1971), Lin et al (1972a, 1972b) and Gupta (1976) have also reported that atleast one of the antigenic fractions contained in the heart of most other mammals viz rabbit, monkey, man and rat respectively, may not be protein in nature as this fraction has been reported to be thermoresistant and is precipitable by alcohol. These results are not inconsistent with the possibility that his fraction may be polysaccharide in nature and



Schematic representation of antigens contained in the heart of different species of animals.

Centre = Animal immunized.
Inner circle = Species of immunizing animal.
Middle circle = Number of cardiac chambers in corresponding immunizing species.
Outer circle = Number of antigenic systems contained in the heart of particular species of animal

SUMMARY AND CONCLUSIONS

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In the present study, various antigenic systems contained in the heart of different species of animals viz. Fish, Frog, Tortoise, Hen, Bat and Pig have been investigated, and attempts have been made to analyse the phylogenic development of these antigens and also to find out any possible immunological correlation with reference to number of cardiac antigens and complexity of development of heart, in these animals as the heart became from two to four chambered right from pisces to mammalia, during the course of phylogenic development.

Rabbits were immunized heterologously with cardiac antigens (suspension of heart extract and complete Freund's adjuvant in equal proportion) of various species of animals viz, Fish, Frog, Tortoise, Hen, Bat and Pig, once a week for six weeks consequetively. Sera from rabbits were collected ten days after the last injection and tested for the presence of anticardiac antibodies using agar gel diffusion (AGDP), immuno-electrophoresis (IEP) and immuneprecipitation electrophoresis (IPE) techniques.

Sera of all the rabbits immunized with different cardiac antigens reacted with the development of variable number of precipitin bands against corresponding cardiac antigen in AGDP, IEP and IPE techniques. Thus anti-fish-heart

antiserum and anti-tortoise-heart antiserum reacted with the corresponding cardiac antigens to give only two precipitin bands by all these techniques whereas the anti-frog-heart antiserum, anti-hen-heart antiserum and anti-bat- heart antiserum gave three precipitin bands against the corresponding cardiac antigens; the pig heart extract gave four to five precipitin bands against corresponding cardiac antigen.

The cross-reactivity of anti heart antisera of various animal species with the cardiac antigen of different animals as well as in reverse experiments the anti-cardiac antisera reacted only with the cardiac antigen of the corresponding animal species (immunizing species), and not with the cardiac antigen of any other species of animals. It would suggest that common cardiac antigens are not shared by these animals and the antigenic system contained in heart of different species of animals are species specific.

The results of all the three techniques viz. AGDP, IEP and IPE were identical in all experiments, therefore in some of the further experiments only one or two of these techniques were used for the detection of cardiac antigens.

It is conceivable from the results obtained after exposure of various heart antigens to various temperatures that fish heart antigens are heart labile and are completely

destroyed at 70°C or more within 30 minutes. The frog heart antigen could withstand 70°C temperature upto 30 minutes whereas it became inactive at 80°C temperature or more. The tortoise heart antigen was extremely heat labile and destroyed even after exposure to 55°C temperature. Hen heart antigens were much resistant to heat as evident from the results that it could withstand temperature upto 100°C, reactivity of hen heart antigen, though faint, remained intact even after exposure to 100°C temperature. The heart antigens contained in bat were moderately heat labile and were found inactive at 70°C temperature or more. The pig heart antigens are the most resistant to higher temperature and the reactivity of some of the antigenic fractions remained intact even at 100°C temperature.

The heart extracts variously exposed to pH, on cross reactivity with corresponding anti heart antiserum in AGDP, IEP and IPE techniques revealed that fish heart antigens were optimally active at moderately alkaline pH 7.2, 8 and 9.5, whereas the antigenic reactivity was lost at a low pH of 4.5, Tortoise heart antigen was much sensitive to change in pH; it was labile at low (4.5 and 6) as well as high pH (8 and 9.5) and was found to be optimally active only at pH 7.2. The hen heart antigen was moderately active at acidic pH 4.5 and 6, optimaly active at 7.2 and

labile at pH 9.5. The bat heart antigenic systems were moderately active at pH 4.5, 6 and 9.5 whereas optimally active at pH 7.2. The pig heart antigens were feebly reactive at acidic pH 4.5 and 6 while moderately active at pH 8, and optimally active at 7.2, whereas it was labile at pH 9.5.

The results of cross reactivity of protein fractions obtained after graded salting out with ammonium sulphate and the corresponding anticardiac antiserum revealed that the fish heart antigens were partially isolated at 40% and 60% saturation with ammonium sulphate whereas at 100% saturation all components were isolated indicating thereby that fish heart antigens are protein in nature. The frog heart antigens were partially isolated at comparatively lower saturations with ammonium sulphate whereas all components were precipitated at 100% saturation, which indicates that these are also protein in nature. All the antigenic components contained in tortoise, hen and bat heart were also precipated at 100% saturation with ammonium sulphate which indicates that all the antigenic fractions contained in tortoise, hen and bat heart are also protein in nature. However, even at 100% saturation with ammonium sulphate only three out of 4 to 5 antigenic fraction of pig heart could be isolated which

indicated that beside three protein antigens, atleast one to two antigenic fractions contained in pig heart could be other than protein.

Previous studies suggested that heart polysaccharide is antigenic acting like hapten (Chaturvedi, Gupta and Mehrotra et al 1971, Gupta 1977). The results reported here are not inconsistent with the possibility that one to two antigenic fractions of pig heart are polysaccharide in nature.

The relevant literature on the subject has been reviewed and the findings of the present work discussed in light of it.

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